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SPECTROPHOTOMETRIC DETERMINATION OF BLOOD CHOLESTEROL BY MEANS OF OXIDE OF SILICA.

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DURING the course of investigations on blackwater fever in Greece, difficulties were encountered in carrying out accurate estimations of blood cholesterol. The chaotic state of the literature on the subject of cholesterol metabolism and estimation make it appear likely that the methods generally used for the determination of this substance are not sufficiently accurate ; and the observations of one worker cannot be regarded as comparable to those of others unless the utmost precautions are taken to control such variable factors as time, temperature and light, and the freshness of the cholesterol standard. Great differences of opinion exist on such a relatively straightforward matter as the normal cholesterol level in man, as is shown in Table I, in which the normal concentrations as stated by various authors are given. Further, it is well known that the use of different methods of estimation give widely varying results when applied to the same blood, as shown in Table II.

It seems, then, that the Liebermann-Burchard reaction generally used for the estimation of cholesterol is apt to give very discordant results in the hands of different workers, most being agreed that checks nearer than 15 to 30 p.c. are not possible ; Kirchgessner (1934), after extensive trials of the colorimetric methods, substituted the more laborious but accurate gravimetric.

To some extent this inability to get satisfactory checks is due to the development of a yellow discoloration in the unknown blood extract, making comparison with the standard solution of cholesterol in chloroform a matter of very great difficulty, and rendering the results obtained of very doubtful value. In our experience several estimations on the same blood are quite likely to give discrepancies that range from 10 mg. to 70 mg. p.c., when the Myers and Wardell plaster of Paris method is used. Andes (1935) has recently shown what we have repeatedly found to be the case, namely, that unless the freshest plaster of Paris is used, the results obtained are likely to be so wide of the mark as to be useless. The moisture of the atmosphere is an important factor in determining the state of hydration of the plaster, and hence the amount of cholesterol extracted from the blood and plaster mixture. Lower values are likely to be obtained if it is high, and *vice versa*. The differences

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are so great as to render the results worthless unless precautions are taken to check the state of the plaster, as shown in Table III.

TABLE I.

Authors.	Whole blood (mg. p.c.).	Plasma (mg. p.c.).	Serum or red blood-cells (mg. p.c.).
Hawk and Bergein ('Phys. Chem.', 10th ed.)	140–180	..	150–180
Petersen and Levinson (1930)	160–245	..	..
Rosen and Krasnow (1930)	111–193	..	..
Gorham and Myers (1917)	130–190	..	..
Bloor (1916, 1928)	200–230	..	..
Mathews ('Phys. Chem.', 1930)	150	..	..
Beaumont and Dodds ('Recent Advances in Medicine,' 1934)	150–180	..	..
Sackett quoted by Ross (1932)	150–190	..	..
,, (1925, *a*)	117–288	..	..
Myers ('Chem. Anal. Blood,' ed. 1924)	159	170	170
Andes (1935)	80–198	..	..
Peters and Van Slyke ('Quant. Clin. Chem.', vol. i)	100–230	100–230	100–230

Normal cholesterol values for man as quoted in the literature by various authors.

TABLE II.

Method.	Whole blood (mg. p.c.).
Myers and Wardell (Harrison, 'Chem. Methods in Clin. Medicine')	100–200
Bloor ,, ,, ,, ,, ,,	167–255
Digitonin ,, ,, ,, ,, ,,	78–227

Values for cholesterol of man as obtained by various methods (Harrison).

TABLE III.

Blood sample.	Old plaster (mg. p.c.).	New plaster (mg. p.c.).
A. Serum } Same blood {	108*	201*
,, } Same blood {	105*	199*
B. Serum } Same blood {	161*	213*
,, } Same blood {	152*	219*
C. Whole blood	184*	213*
D. ,,	110	220
E. ,,	140	238
F. ,,	170	215
G. ,,	85	130
H. ,,	100	145
I. Serum	150	187
J. ,,	170	210

* Taken from Andes.

Cholesterol values obtained on the same blood by the use of fresh and old plaster of Paris.

The difficulty of getting satisfactory checks is always more pronounced when a brownish tinge is present in the unknown blood extract. But when the match between the standard and the unknown was a good one, the difference between two estimations was frequently 10 mg. to 20 mg. p.c., even though the plaster was known not to be hydrated, this was only partly accounted for by insufficient extraction, although this had proceeded for several hours.

The brown discoloration is rather less frequent if Sackett's (1925) method is used, provided the precautions recommended by Rabinowitch (1929) are observed. In the Myers and Wardell method there is frequently a yellow tinge in the chloroform extract before the addition of the acetic anhydride and sulphuric acid, indicating that these reagents cannot be regarded as responsible. Nor can overheating be regarded as the cause, since of several samples extracted on the same electric water-bath, some developed the yellow colour, while others did not. During the course of our work we tried all the various methods suggested in the literature for the elimination of the offending colour, but conclude that none can be regarded as a certain and never-failing means of preventing its development. We tried such methods as the following for preventing the brown discoloration: (1) The avoidance of overheating; (2) allowing the colour development to take place at 5° C. (Mirsky and Bruger, 1923); (3) using the purest reagents (Heilmeyer, 1933; Urbach, 1932); (4) exclusion of water from the extract and reagents (Myers and Wardell, 1918); (5) washing the extract with anhydrous sodium sulphate (Mueller, 1916); (6) addition of oleic and palmitic acids to the standard (Cantoni, 1930); (7) permitting the green colour to change to yellow by incubating at 20° C. for 24 hours (Schube, 1932); (8) using the tungstic acid precipitates of de Toni (de Toni, 1926); (9) using a red glass filter on the colorimeter (Bloor, 1928). The use of some of the above methods was not only without effect, but often produced fantastic results.

We are unable to suggest to what this discoloration is due, whether to substances present in some bloods, or to impurities, or other factors, in the reagents; we are inclined to think the former, but have no definite evidence on this point. We cannot agree with the statement of Luden (1915–1916) that the discoloration is commoner in bloods from icteric patients. A large number of our estimations have been made on the bloods from cases of blackwater fever, in which the bilirubin content was frequently anything from 1 mg. to 50 mg. p.c., and quite often the match between the standard and the unknown was an excellent one. We do not think that the yellow colour which develops in the standard when allowed to remain for some hours is the same as that which appears in the blood extract, since that in the blood is present before the addition of the acetic anhydride and sulphuric acid. The yellow colour that develops in the standard is due to a continuation of the reaction to its termination, the final product of which is a resinous substance (Bloch, 1929).

It is this substance that Schube (1932) has suggested might be used for colour comparison. But the yellow colour is, in our experience, as variable as the green, and in view of our spectrophotometric determinations, it would seem that the absorption values for the yellow colour of the standard and the unknown are not the same. Bloch (1933) has pointed out that the colour

change which occurs in the Liebermann-Burchard reaction is a progressive one, and that after 15 minutes only three-quarters of the cholesterol present in the solution has developed its colour, the remainder forming later. From spectrophotometric work done in this laboratory, it would appear that no definite statement can be made with regard to the amount of cholesterol that may have developed its colour after 15 min.; in some samples the maximum absorption value was reached after 10 min., while in others 30 min. did not suffice for its maximum production.

TABLE IV.—*Spectrophotometric Estimations of the Variations in the Amount of Cholesterol Present in a 135 mg. p.c. Solution of Cholesterol in Chloroform after a Lapse of Varying Periods of Time. (Light and Temperature Controlled.)*

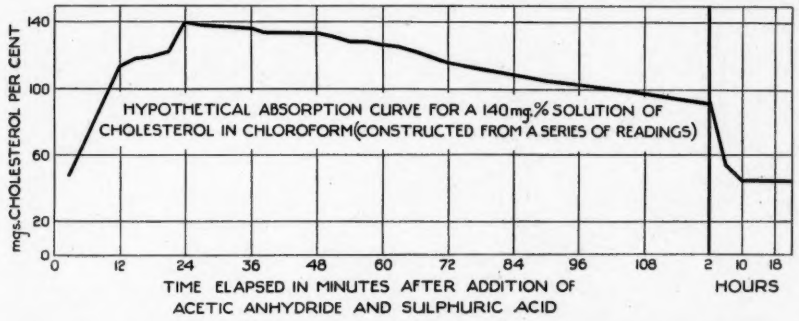
Period of time elapsed. hr. min.	Temperature.	Mg. p.c.	Period of time elapsed. hr. min.	Temperature.	Mg. p.c.
3	18° C.	40	54	18° C.	120
6	,,	64	57	,,	116
9	,,	88	1 00	,,	116
12	,,	102	1 3	,,	113
15	,,	105	1 6	,,	113
18	,,	111	1 9	,,	113
21	,,	120	1 30	,,	104
24	,,	123	1 45	,,	99
27	,,	129	2 00	,,	91
30	,,	129	2 15	,,	88
33	,,	133	2 40	,,	83
36	,,	126	3 15	,,	76
39	,,	126	5 00	,,	62
42	,,	126	6 00	,,	55
45	,,	123	7 00	,,	45
48	,,	123	10 00	,,	45
51	,,	123	24 00	,,	45

TABLE V.

Cholesterol (mg. p.c.).	Maximum value attained after (minutes).	Maximum value remained stable for (minutes).	Lowest value attained after (hours).	Temperature throughout the reaction (° C).
140	18	9	24	17
140	32	8	48	17
135	30	—	48	17
135	35	6	72	17
135	33	3	10	18
125	20	5	16	17
125	20	10	40	17
125	12	4	35	17
200	23	18	24	17

Spectrophotometric estimations taken as in Table III, but for varying concentrations of cholesterol.

In Table IV are shown the absorption values in mg. p.c. of a 135 mg. p.c. solution of cholesterol in chloroform. In Table V similar information is summarized for solutions varying in strength from 125 mg. p.c. to 200 mg. p.c. The readings were taken every 3 minutes up to 24 hours, after the addition of the acetic anhydride and sulphuric acid. The absorption values in both these tables were obtained by the use of a Pulfrich stufenphotometer. This instrument is capable of measuring the amounts of light transmitted through monochromatic spectral filters (ranging in wave-length from 720–430 mμ) that differ in intensity by less than 0·001 p.c. In estimating the extinction co-efficients of these solutions we used a cell having a stratum thickness of 0·5 cm., and a filter having a wave-length of 610 mμ. Our readings were taken against distilled water, since it had been shown that a solution of acetic anhydride and sulphuric acid in chloroform had no absorption in the visible region of the spectrum. In all the estimations the temperature was controlled, and the colour development took place in complete darkness, except for the



brief periods during which the readings were being taken, when a 100-watt Wotan Nitra light of 8 amp. was used. The exposure to this light lasted in every case for 20 sec. From these tables it will be seen that the absorption increases to its maximum point in an average time of about 25 min., remains steady for a variable period, averaging about 6 min., and then begins slowly to decrease. In some cases it was found that the maximum colour value was not reached till after a lapse of 48 min., while in others it was reached after a lapse of only 18 min. The same variability was found to exist with the persistence of the colour after it had reached its maximum. In some instances the colour remained stable for 18 min. and then began to decrease, while in others a fall set in after 3 min. With higher temperatures the rate at which the colour develops is more rapid. The information is summarized in the accompanying graph.

There is some evidence, which will be presented in later papers, that the rate at which the standard solution develops its maximum absorption is not the same as that for blood extracts; in other words, the temperature, time and light relationships are not the same for the standard and the unknown. In this connection it is of interest to note that Reinhold (1934) has claimed that the discrepancies between the time development of colour in pure

solutions of cholesterol and chloroform extracts of human blood may be due to the existence in blood of an isomer of cholesterol; he further points out that cholesterylpalmitate (prepared by heating cholesterol with palmitic acid) differs from cholesterol in its reactions with acetic anhydride and sulphuric acid. Cantoni (1930) has further pointed out that if palmitic acid be added to the standard when there is a yellow colour present in the unknown, it renders the standard comparable with the unknown.

Another factor of importance is the deterioration of the standard solution of cholesterol in chloroform. Photometric readings taken on a 100 mg. p.c. solution have shown that after three weeks the absorption value is equivalent to 85 mg. p.c. And this rate of deterioration is not the same for all solutions of the same strength, even when kept under similar laboratory conditions.

Fidler (1934) has remarked on this colour instability as a source of error, and has suggested that the unknown solution of cholesterol should be transferred to the colorimetric cup two minutes after the addition of the acetic anhydride and sulphuric acid; and continuous readings taken against a naphthol green B standard, until the maximum cholesterol value has been attained. This method of taking the readings certainly eliminates the necessity of controlling the light, time and temperature factors, but it might introduce an evaporation error, which in the tropics might not be inconsiderable, where temperatures run from 35° to 45° C. Provided that this evaporation error can be controlled, we feel that Fidler's method is a step forward in the many attempts to make the colorimetric estimations of blood cholesterol really quantitative. It further has the advantage of rendering the observations of one worker comparable with those of another. It still, however, leaves the yellow colour problem unsolved. With a Pulfrich photometer the evaporation can be completely controlled, since the cells supplied with this instrument can be sealed with ground-glass covers, and we have used the method with the greatest success. With the ordinary colorimeter, however, it is difficult or impossible to control evaporation.

For the reasons that the standard solution of cholesterol does not develop its maximum colour at the same rate as the blood extracts, and that it tends to deteriorate with keeping, it is better to use an artificial standard, made by taking 1 c.c. of a 1 p.c. aqueous solution of the dye and adding it to 19 c.c. of distilled water. This has a colour value equivalent to that produced by 0·4 mg. of cholesterol in 5 c.c. of choloroform. It must, of course, have its colour value checked against a freshly prepared solution of cholesterol. It appears to keep indefinitely if stored in a dark bottle. Some brands of naphthol green B* have a bluish tinge in them, which makes comparison with the ununknown difficult, and often arbitrary. With a photometer, of course, standard solutions can be dispensed with altogether, with a saving of time and an increase in accuracy.

Some authors have experienced difficulty in obtaining a homogeneous mixture of blood and plaster of Paris, considerable clogging and caking taking place, especially if too much blood is used. On this account, and for the more important reason of the inconstancy of the results obtained when plaster is used, we have substituted oxide of silica. Not only does this substance absorb

* The dye which we used was supplied to us by British Drug Houses, London.

the blood without caking, but its absorptive power is four or five times greater than that of the plaster; less, therefore, is needed, with a consequent lower dilution of the blood. In addition it gives almost constantly higher extraction results than either the plaster or the filter paper method of Leiboff, indicating that the "lightness" and "openness" of the blood and silica mixture gives better percolation than the plaster. Further, when we have extracted through silica we have rarely or never been troubled with the yellow discoloration so common with the plaster. In Table VI are given the cholesterol values obtained on the same sample of blood by the use of silica, plaster of Paris, and the Leiboff method.

TABLE VI.

Myers and Wardell plaster (mg. p.c.).	Myers and Wardell silica (mg. p.c.).	Leiboff filter-paper (mg. p.c.).
152	187	128
160	197	150
130	187	123
105	140	185
145	150	142
150	173	134
..	150	102
..	118	91
..	187	155
..	115	Same blood: 98, 100
..	131	,, 100, 109
..	Same blood: 152, 150	,, 152, 149
..	,, 138, 130	,, 113, 95

Cholesterol values obtained by the use of Myers and Wardell plaster and silica and the Leiboff filter-paper methods.

During the process of extraction it is wise to see that there is always a shallow layer of chloroform above the blood and silica mixture; if this is not attended to the extraction may be anything but complete, because the slow dropping of the chloroform in one spot may, and often does, leave a certain amount of cholesterol behind in that part of the mixture that has not been sufficiently percolated by the chloroform.

In the Myers and Wardell procedure round-bottomed flasks are recommended; it is better to substitute instead ordinary hard glass test-tubes, graduated at the figure it is intended to use as a dilution factor. (We use 15 c.c.) In this way it is possible to avoid the last transference from the extraction apparatus to the tubes where the acetic anhydride and sulphuric acid are to be added, and to carry out the extraction and coloration in the same

tube. Leiboff (1924) and Andes (1935) have both devised very convenient extractors, whereby both the extraction and coloration can be carried out in the same tube; but both these authors use 0·25 c.c. of blood only, and we feel that in a method already none too accurate the use of such small amounts of blood is not conducive to extreme accuracy.

Lastly it is wise to standardize the amounts of acetic anhydride and sulphuric acid added to the extract, since the rapidity and intensity of the colour development is to some extent dependent upon the relative amounts of these two reagents to the total amount of the extract. We have used 4 c.c. of acetic anhydride, and 0·4 c.c. of concentrated sulphuric acid, to 15 c.c. of chloroform extract. We have always used Merck's or Kahlbaum's pro-analysis material, but no doubt any good grade chemicals would serve. We also took the precaution of keeping our chloroform over CaCl, but this is probably not necessary.

In a future paper the spectrophotometric method which we have used will be reported; here only an outline will be given of the method used for the final determination.

METHOD.

0·5 c.c., 1 c.c. or 2 c.c. of serum, whole blood, or plasma, are pipetted on to 1, 2 or 3 g. of silica, which can be roughly weighed or estimated. An evaporating basin is a convenient receptacle. The blood is allowed to drop into small "wells" made in the silica, care being taken to see that none comes into contact with the walls of the basin.

After half an hour or so the blood will have dried sufficiently to permit extraction. (We usually keep ours in a large desiccator until we have some 8 or 10 on hand and then extract altogether on a large electric water-bath; thus very complete drying is ensured, and mixing is a matter of the greatest ease.) The mixture of blood and silica is then transferred to the extraction thimble, care being taken to make the transference quantitative. About 10 c.c. of good grade chloroform is added to the tube of the extraction apparatus, and the whole assembled, and extracted for 2 to 4 hours, care being taken to see that a layer of chloroform is always above the mixture; a temperature of 70° to 75° C. will usually ensure this. See that the thimble does not dip into the chloroform (that the test-tube is long enough to enable the thimble to be suspended well above the chloroform); in this way endless time is saved over washings.

When the extraction is complete, dismantle the apparatus; make up the extract to the dilution mark (say 15 c.c.). Add 4 c.c. of high-grade acetic anhydride, and 0·4 c.c. of concentrated sulphuric acid. Place in a dark incubator at 20° C. for 25 minutes. If an incubator is not available, any constant temperature device will do, such as a warm water or oil bath placed in a dark cupboard. The time and temperature, and light, must always be rigidly controlled for every estimation, and remain the same for every series of experiments. Compare in the colorimeter, and calculate the amount of cholesterol according to the usual formula:

$$\frac{S}{U} \times \text{concentration of standard} \times 100 = \text{mg. p.c.}$$

If a photometer is available no standard will be required; and if the Fidler method is used, an artificial standard is necessary of naphthol green B. With the Pulfrich stufenphotometer a convenient stratum thickness is 0·5 cm. and a filter having a wave-length of 610 mμ. The unknown drum-head is set at 100 p.c., and the other drum rotated until the amount of absorption, as shown by equality of light in the eye-piece, is the same. One series of readings is taken with the extract on the left; the extract is then interchanged and a series of readings taken with it on the right, in order to compensate for any differences in the acuity of the two eyes.

The cholesterol concentration is read off directly from a previously constructed concentration curve. It is convenient to use single logarithm paper for making up the concentration curve, as then drum readings can be read off directly from the curve, and the use of extinction coefficient tables avoided. On the curve drum readings are plotted as ordinates, and milligrammes as abscissæ.

SUMMARY.

Certain criticisms are made of the present methods of determining blood cholesterol by means of the Liebermann-Burchard reaction, and suggestions made for overcoming some of them.

Inability to get satisfactory checks when the Myers and Wardell method is used is attributed to variations in the state of hydration of the plaster of Paris.

Determinations of the instability of the green colour have been made spectro-photometrically, and the conclusion is reached that no statement can be made regarding the rate of its formation or disappearance as suggested by Bloch.

A discussion of the formation of the yellow colour in the blood extract is given, with the means suggested for its elimination.

A modified method is given using oxide of silica instead of plaster of Paris, and a photometer instead of a colorimeter.

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COMPLEMENT-FIXATION WITH VACCINIAL ELEMENTARY BODY SUSPENSIONS AND ANTIVACCINIAL RABBIT SERUM.

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ALTHOUGH complement-fixation by crude vaccinia suspensions in the presence of immune sera has been demonstrated by many workers (see Parker and Muckenfuss, 1933, for bibliography), the exact nature of the antigenic factor has not yet been elucidated.

In view of recent work supporting the view that the "elementary body" represents the virus of vaccinia, it appeared valuable to determine the *role* of "elementary body" suspensions as complement-fixing antigens.

METHODS.

The technique adopted was a modification of that described by Mackie and Finlayson (Finkelstein) (1930) for demonstrating complement-fixation with normal sera and bacterial suspensions. It appeared advisable to use a technique in which both variations in complement and serum dilutions would help to detect weak fixation effects as demonstrated by other workers. Preliminary experiments showed that mixtures of complement, antigen and serum which were allowed to stand overnight at 0° C. demonstrated good fixation of complement on addition of a hæmolytic system of sensitized 3 p.c. sheep corpuscles. It was noted that if antigen and serum remained in contact overnight, when complement was added and the mixture allowed to stand for two hours, fixation of complement was not complete. It would therefore appear that the complement is fixed very slowly by the antigen-antibody complex.

Antigens.—Suspensions of elementary bodies were prepared by two methods: (*a*) (A modification of Ledingham's method.) Vesicle formation was obtained by rubbing the skin of a freshly-shaved rabbit with calf-lymph. The animal was killed four to five days afterwards and the vesicle contents collected with a blunt scalpel. This lapine was ground in a mortar with 10–20 c.c. distilled water (the volume of water varied with the yield of lapine), and spun for five minutes at 2000 r.p.m. to deposit gross material. The supernatant fluid was spun at high speed (10,000–15,000 r.p.m.) for 30 minutes and the deposit retained. This deposit was resuspended in the original volume of saline, and again centrifuged for 30 minutes at 10,000–15,000 r.p.m. The supernatant was discarded, and the deposit resuspended in saline to the original volume.

(*b*) This method is essentially that described by Craigie (1932). After the final centrifugation on the angle centrifuge, the deposit was resuspended in the

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original volume of saline and spun on a high-speed centrifuge for 30 minutes at 10,000–15,000 r.p.m. The supernatant was discarded, and the deposit resuspended in the original volume of saline.

Suspensions of elementary bodies prepared by both methods were examined for activity by intradermal inoculation of rabbits. Decimal dilutions were made using separate pipettes for each dilution. 0·2 c.c. of each dilution was inoculated intradermally into the skin of a shaven rabbit, and readings were made 4 and 7 days after inoculation. The presence of large numbers of elementary bodies was verified by staining of films and also by dark-ground illumination.

Sera were obtained from rabbits infected with vaccinia, and then hyperimmunized by subcutaneous and intravenous inoculations of "lapine". Sera were heated to 55° C. for 30 minutes before use.

Complement.—Pooled sera from three or more guinea-pigs separated from the clot after 24 hours was used. The minimum hæmolytic dose of complement was determined by incubating varying volumes of guinea-pig serum with 0·25 c.c. of the hæmolytic system for one hour.

Hæmolytic system.—Thrice-washed sheep cells were used in 3 p.c. concentration in 0·85 p.c. saline. Excess of rabbit and sheep amboceptor was added (5 sensitizing doses), and used for determination of complement unit one hour after preparation.

Test.—The quantities found most suitable for experiment were antigen 0·1 c.c., serum dilutions 0·1 c.c., and 0·25 c.c. hæmolytic system. Complement was varied, using the volume which produced complete hæmolysis of 0·25 c.c. sensitized sheep-cells as a unit. Antigen, serum and complement were mixed in that order, shaken and placed in the ice-box at 0° C. for 18 hours. The mixture was removed from the ice-box and allowed to stand at room temperature for 1 hour and a unit volume of hæmolytic system added to each tube. The tubes were then incubated at 37° C. and readings made after 1 hour.

Controls.—The complement-fixing activity of the serum used was controlled in each test by determining the fixing power of a 1 in 10 dilution of the serum.

The activity of the antigen was also controlled in the tests by determining the fixing power of each antigen in the absence of the hyperimmune serum.

EXPERIMENTAL.

At the commencement of this work great difficulty was experienced owing to the frequent occurrence of "zonal" reactions. On investigation it was found that these reactions occurred when certain concentrations of antigen and hyperimmune serum were used, and in order to obtain experimental data uncomplicated by "zonal" effects, it was found necessary to carry out preliminary titrations with dilutions of antigen and the hyperimmune serum intended for use in each experiment. Thus, Table I illustrates the effect of dilution of antigen in abolishing the zonal effect. It should be noted that this effect occurs only when the antigen possesses complement-inhibiting properties, and that dilution of the antigen produces an uncomplicated, true fixation of complement in the presence of the hyperimmune serum.

It should be emphasized that the tables included in this paper have been

TABLE I.—*Effect of Dilution of Antigen. Vaccinia Elementary Body Suspension and Hyperimmune Rabbit Serum.*

Antigen.	Serum.	Doses of complement fixed. 2	4	6	8	Antigen control. 2	4	Serum control. 2	4
Undiluted	1/10	++	0	...	...	+++	++	0	...
	1/20	++	++	0	...	...	...	...	...
	1/40	++	+++	++	0	...	...	...	...
	1/80	+++	+++	+++	+++	...	...	...	...
	1/160	+++	++	++	+++	...	...	...	...
Diluted 1/10	1/10	+++	++	0	...	0	...	0	...
	1/20	+++	++	0	...	...	...	...	...
	1/40	+++	0	...	...	...	...	...	...
	1/80	+++	0	...	...	...	...	...	...
	1/160	0	...	...	...	...	...	...	...

+++ signifies no hæmolysis or almost complete fixation of complement.
++ signifies distinct hæmolysis. Some fixation of complement.
0 signifies complete hæmolysis. No fixation of complement.

selected from a large number of experiments, in many of which anti-complementary or fixation effects produced by antigen alone rendered the results unsuitable for illustration purposes.

An attempt was made to analyse the antigenic capacity of suspensions of elementary bodies by means of the high-speed centrifuge. Suspensions of elementary bodies were spun at various high speeds and the deposits suspended in the original volume of saline. The deposits and supernatant fluids were then tested for antigenic activity in fixing complement.

Table II (*a*, *b* and *c*) illustrates the complement-fixing activity of suspensions of elementary bodies, of Berkefeld V filtrates of these suspensions, and also of the supernatant fluids obtained by centrifugation of the Berkefeld V filtrates. The complement-fixing activity of the deposits after centrifugation resuspended in the original volume of saline is also illustrated. The centrifugation was carried out at 15,000 r.p.m. for 30 minutes in Experiments 2 (*a*) and 2 (*b*), and here it will be observed that all activity was removed from the supernatant fluid.

TABLE II (*a*).—*Vaccinia Elementary Bodies and Hyperimmune Rabbit Serum.*

Serum dilutions.	Degree of fixation with antigen. Elementary body suspension.	Berkefeld V filtrate of elementary body suspension.	Supernatant V filtrate.	Deposit V filtrate.	Serum control.
1 in 10	+++	+++	−	++	−
,, 20	+++	+++	−	++	
,, 40	+++	++	−	+	
,, 80	++	+	−	+	
,, 160	+	±	−	−	
Antigen control	−	−	−	−	

TABLE II (*b*).—*Vaccinia Elementary Bodies and Hyperimmune Rabbit Serum.*

Serum dilutions.	Degree of fixation with antigen. Elementary body suspension.	Berkefeld V filtrate of elementary body suspension.	V filtrate supernatant after 15,000 revs. for 30 min.	V filtrate deposit after 15,000 revs. for 30 min.	Serum control.
1 in 10	++++	++	−	+	−
,, 20	++	+	−	+	
,, 40	++	+	−	+	
,, 80	++	+	−	+	
,, 160	+	+	−	−	
Antigen control	−	−	−	−	

TABLE II (*c*).—*Vaccinia Elementary Bodies and Hyperimmune Rabbit Serum.*

Serum dilutions.	Elementary body suspension.	Berkefeld V filtrate of elementary body suspension.	V filtrate supernatant.	V filtrate deposit.	Serum control.
1 in 10	+++	++	+	+	−
,, 20	++	++	+	+	
,, 40	++	+	+	+	
,, 80	++	+	+	±	
,, 160	+	−	±	−	
Antigen control	−	−	−	−	

V filtrate supernatant = supernatant fluid obtained after spinning Berkefeld V filtrate.
V filtrate deposit = deposit from V filtrate supernatant resuspended in the original volume of saline.
+++ signifies fixation of six units of complement.
++ signifies fixation of four units of complement.
+ signifies fixation of two units of complement.
± signifies fixation of less than two units of complement.
− signifies fixation of no units of complement.

This nomenclature applies also to all the following tables.

In Table III is illustrated the complement-fixation produced by suspensions of elementary bodies, and the reduction in this effect due to centrifugation for 30 minutes at 10,000 r.p.m. The supernatant was again centrifuged for 30 minutes at 10,000 r.p.m., and it will be observed that all the complement-fixing activity was removed by this second spinning. The activity was, however, present in the deposit when resuspended in its original volume of saline.

The effect of filtration on the complement-fixing activity of elementary body suspensions is illustrated in Table IV. It will be seen that whereas the activity of the antigen is reduced but not abolished by filtration through Berkefeld V and N filters, filtration through a Seitz filter completely removes it.

Finally, in order to identify more accurately the antigen with vaccinia virus, filtrates of elementary body suspensions through gradocol membranes (Elford and Andrewes, 1932) were made and tested for complement-fixing

TABLE III.—*Vaccinia Elementary Body Suspension and Hyperimmune Rabbit Serum.*

Serum dilutions.	Degree of fixation with antigen. Elementary body suspension.	Supernatant (1) after spinning bodies 30 min. at 10,000 r.p.m.	Deposit re-suspended after spinning bodies 30 min. at 10,000 r.p.m.	Supernatant (2).	Deposit (2).	Serum control.
1 in 10	++++	++	++	−	+	−
,, 20	+++	+	+	−	+	
,, 40	+++	+	+	−	+	
,, 80	++	+	+	−	−	
Antigen control	−	−	−	−		

Supernatant (2) = supernatant fluid obtained after spinning (1) for 30 min. at 10,000 r.p.m.
Deposit (2) = resuspended deposit obtained after spinning supernatant (1) for 30 min. at 10,000 r.p.m.
Resuspended in original vol. of supernatant (1).

TABLE IV.—*Vaccinia Elementary Body Suspension with Hyperimmune Rabbit Serum.*

Serum dilution.	Degree of fixation with antigen. Elementary body suspension.	Berkefeld V filtrate of elementary body suspension.	Berkefeld N filtrate of elementary body suspension.	Seitz filtrate of elementary body suspension.	Serum control.
1 in 10	++++	+++	++	−	−
,, 20	++++	+++	++	−	
,, 40	+++	+++	+	−	
,, 80	+++	+++	+	−	
Antigen control	−	−	−	−	

TABLE V.—*Vaccinia Elementary Body Suspensions with Rabbit Hyperimmune Sera* (A and B).

Serum dilutions.	Degree of fixation with antigen. Elementary body suspension.	Bodies filtered through 0·65 mμ membrane.	Filtrate of 0·65 mμ membrane, passed through 0·15 mμ membrane.	Serum control.
(A) 1 in 10	+++	+++	−	−
,, 20	+++	+++	−	
,, 40	++	++	−	
,, 80	++	+	−	
(B) 1 in 10	+++	+++	−	−
,, 20	+++	++	−	
,, 40	++	++	−	
,, 80	++	+	−	
Antigen control	−	−	−	

activity. A filtrate containing elementary bodies was obtained after passing a suspension through a collodion membrane of pore size 0·65 mμ, and a portion of this fluid was passed through a membrane of pore size 0·15 mμ. This filtrate and some of the 0·65 mμ membrane filtrate, together with the original suspension of elementary bodies, were tested for complement-fixing activity. These filtrates were kindly prepared by Dr. Elford. The results of the experiment, with two filtrates and two sera, are shown in Table IV. It will be observed that some reduction in the complement-fixing activity of the suspension of elementary bodies occurred after filtration through the membrane of 0·65 mμ pore size. This active filtrate, however, was rendered quite inert with respect to fixation of complement, by filtration through a membrane of 0·15 mμ pore size.

DISCUSSION.

Although many workers have produced fixation of complement in the presence of vaccinia skin or brain suspensions, the antigenic agent in these suspensions has so far proved elusive. The observations of Gilmore (1931), and also of Bedson and Bland (1929), have apparently ruled out the bacterial factor, which had been implicated by Schulz and co-workers (1928). On the other hand, while Thompson, Hazen and Buchbinder (1932) state that "the evidence indicates that this fixation is a process specific for vaccinal virus tissue", Parker and Muckenfuss (1933), working with crude "lapine", report that active virus in the antigen is not necessary for fixation, and that vaccinia material which had been passed through a Berkefeld N candle and in which no virus could be demonstrated would still fix complement. Craigie (1934), in a short abstract, has claimed to obtain complement-fixation with Seitz filtrates of suspensions of crude lapine.

The observations of Ledingham (1931, 1932), and of Craigie (1932), that vaccinia elementary body suspensions are agglutinated by hyperimmune vaccinia serum, suggest that these bodies might be antigenic in fixing complement in the presence of anti-vaccinial serum. The observations illustrated show that fixation of complement occurs with suspensions of elementary bodies in the presence of hyperimmune rabbit sera. The filtrates obtained by passing these suspensions through V and N Berkefeld filters also fix complement, and, as Green and Eagles (1931) have shown, Berkefeld V and N filtrates contain vaccinia virus. On the other hand, Seitz filtrates of elementary body suspensions which do not contain vaccinia virus do not fix complement. Experiments with gradocol membranes also indicate that no fixation of complement occurs in the absence of vaccinia virus.*

The experiments carried out with the high-speed centrifuge show that the antigenic activity of a suspension of elementary bodies may be removed by centrifugation, so that the supernatant fluid is inactive, and all the complement-fixing activity is retained in the resuspended deposits of elementary bodies.

* In a later brief communication by J. Craigie and F. O. Wishart (*Trans. Roy. Soc. Canada*, Sect. V, 1934), which appeared since Dr. Finlayson's removal to S. Africa, it is reported that complement-fixation occurs with Seitz filtrates of lapine containing the specific precipitable substance of vaccinia, as well as with the pure elementary bodies. Dr. Finlayson's work is concerned with elementary body suspensions only and filtrates of such (J. C. G. L.).

This work demonstrates that the complement-fixing agent of elementary body suspensions is closely associated with these elementary bodies, and is removed by high-speed centrifugation. The agent is also retained by Seitz filters and by gradocol membranes of 0·15 mμ pore size.

SUMMARY.

(1) Suspensions of " elementary bodies " of vaccinia virus fix complement in the presence of vaccinia hyperimmune rabbit sera.

(2) The complement-fixing activity of the suspensions is reduced but not abolished by filtration through Berkefeld V and N filters, and through gradocol membranes of pore size 0·65 mμ.

(3) The complement-fixing activity of the elementary body suspensions is abolished by filtration through Seitz filters and through gradocol membranes of pore size 0·15 mμ.

(4) The complement-fixing activity is removed from elementary body suspensions by centrifugation at high speeds. The antigenic activity is retained in the resuspended deposit of elementary bodies of vaccinia.

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THE PLASMA PHOSPHATASE IN THE VARIOUS TYPES OF JAUNDICE.

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Two years ago Roberts put forward the view that the estimation of blood phosphatase might be used as a diagnostic test in the differentiation of the various types of jaundice (Roberts, 1933). Using his own method (Roberts, 1930) for the estimation of phosphatase, he found that in jaundice due to mechanical obstruction of the bile-ducts the blood phosphatase showed a striking increase (10·9 to 22·9 units); in jaundice of the toxic or infective type the figures were either normal or slightly raised (3·7 to 8·9 units, except for one case of catarrhal jaundice in a child); and in hæmolytic jaundice the results were always normal (2·2 to 5·5 units). Since the serum bilirubin gives a direct Van den Bergh reaction both in obstructive jaundice and in toxic or infective jaundice, it appeared that the estimation of blood phosphatase would prove a useful diagnostic test in differentiating between these two types.

These results are of great interest also in relation to the theory of the Van den Bergh reaction, for if obstruction to the outflow of bile always causes a rise in blood phosphatase, one would expect the retention of phosphatase and of directly-reacting bilirubin to run parallel. But it is possible, in jaundice of the toxic or infective type, to find intense jaundice due mainly or entirely to the presence of directly-reacting bilirubin, associated with normal values for blood phosphatase. Roberts suggested that a revision of the theory of the Van den Bergh reaction might be necessary. The present work was begun soon after the appearance of Roberts's results, with a view to determining whether his findings could be confirmed.

TECHNIQUE.

Oxalated venous blood was collected, using the minimum quantity of oxalate adequate to prevent coagulation. The usual time of collection was 5 to 6 hours after the patients' breakfast. The samples were centrifuged very thoroughly in order to obtain plasma free of leucocytes. Plasma was used for the estimation of both phosphatase and bilirubin.

Plasma bilirubin.—In performing the direct Van den Bergh test, 1 c.c. plasma and 1 c.c. diazo reagent were used. For the indirect test, the technique of Thannhauser and Anderson was employed (Harrison, 1930). For quantitative estimations, the cobalt sulphate standard recommended by Hunter (1930) was used. Where a direct reaction was found, an attempt was made to determine whether the indirectly-reacting pigment was also increased.

This may be done firstly by noting the quality of colour developed within the first few minutes in the direct test, for if the full colour is developed rapidly, it may be presumed that the whole of the pigment has been changed to azo-bilirubin, *i. e.* the reaction is entirely direct. Secondly, a quantitative estimate may be made of the colour developed in the direct and in the indirect test, so as to determine whether the directly-reacting bilirubin accounts for the whole increase above normal. Thirdly, an attempt may be made to separate the two pigments by chloroform extraction. In the writer's experience " mixed " reactions are uncommon; even in toxic jaundice, the directly-reacting pigment usually accounts for almost, if not quite, all the increase above normal. In fact one often finds the figure obtained for total bilirubin in the indirect test slightly lower than that derived from the direct estimation, probably owing to loss of azo-bilirubin on the protein precipitate in the indirect test. Quantitative estimations of bilirubin are only approximate.

Plasma phosphatase.—In the present work the method of Jenner and Kay (1932) has been used. At the outset it was ascertained that in this method the rate of liberation of inorganic phosphate was constant during the incubation period, and that this constancy of rate held for plasma phosphatase values at least as high as 50 units. In these experiments the usual incubation mixture containing 0·25 c.c. plasma in a total volume of 5·5 c.c. was used. With very high phosphatase values it is probably desirable to work with smaller concentrations of plasma.

In using a method of estimation of an enzyme which depends on the measurement of the amount of change produced in a given time, it is important to secure conditions under which the rate of change is constant; for the rate of the reaction is proportional to the concentration of the enzyme, and if the rate is to be measured by a single determination at the end of the incubation, the rate must be constant throughout if the estimation is to be valid. These conditions are not secured in Roberts's method, for his own figures show that the rate of liberation of phosphate falls off during the incubation period (Roberts, 1930). This source of error should not affect his general conclusions; his figures have comparative value, but it is impossible to translate his " phosphatase units " into the terms of any other method.

A word of warning is needed in regard to the stock stannous chloride solution used in the method of Jenner and Kay. Ordinarily this solution keeps for many months in a dark bottle, but occasionally it may deteriorate, and this leads to a failure in colour development affecting especially the high phosphate values, and introducing very serious errors in proportionality of colour to concentration.

The phosphatase figures in this paper refer to plasma, whereas those of Roberts refer to whole blood.

RESULTS.

The results are given in Tables I to V.

The normal range of the plasma phosphatase in the method of Jenner and Kay is from 3·2 to 7·4 units. In a series of miscellaneous diseases (other than diseases of the liver or the skeleton) Jenner and Kay found values ranging from 4·5 to 14·3.

TABLE I.—*Obstructive Jaundice.*

Case.	Age.	Diagnosis.	Duration of jaundice at time of testing.	Phosphatase units.	Bilirubin. Total units.	Bilirubin. Type of reaction.	Confirmation of diagnosis.
1	67	Obstructive jaundice, probably malignant disease (fatal)	15 months	44·7	28	Direct	Clinical data.
2	53	Carcinoma of head of pancreas	4 weeks	40·6	53	„	Operation.
3	64	„ „ „ (fatal)	4 „	120·0	70	„	Clinical data.
4	66	Obstructive jaundice, malignant disease	5 „	26·2	63	„	„ „
5	66	„ „ „ „ (fatal)	6 days 3 months	18·7 23·6	34 +	„ „	„ „ „ „
6	65	Secondary carcinoma of liver	2 weeks	44·3	3	„	„ „
7	..	„ „ „ (fatal)	2 years	120·0	39	„	Operation.
8	52	Carcinoma of stomach; secondary carcinoma of liver	4 months	61·3	40	„	Clinical data.
9	43	Carcinoma of liver (fatal)	11 weeks	26·6	25	„	„
10	40	Carcinoma of ampulla of Vater (fatal)	10 „ 13 months	13·1 43·0	50 40	„ „	.. Post-mortem.
11	41	Carcinoma of bile-ducts	7 weeks	54·0	68	„	Operation.
12	3	Sarcoma of mesentery (fatal)	6 „	46·8	66	„	Post-mortem.
13	54	Stricture of common bile-duct	2 „	16·7	36	„	Operation.
14	54	Lymphadenoma; glands at portal fissure (fatal)	2 months 2½ „	74·2 42·5	4·2 15·0	„ „	.. Post-mortem.
15	54	Cirrhosis of liver with ascites	2 „	21·7	50	„	Clinical data.
16	11	Cirrhosis of liver (fatal)	7 „ 8½ „	39·8 42 0	36 11	„ „	„ „ „ „
17	57	Gall-stones and carcinoma of gall-bladder (fatal)	9 weeks	23·9	133	„	Operation.
18	49	Single cholesterol stone in common bile-duct. Cholecystitis	6 months	60·0	6	„	„
19	63	Cholecystitis and gall-stones	?	27·4	1·2	„	„
20	..	Gall-stone in neck of gall-bladder	?	12·9	1	„	„
21	25	Gall-stones in gall-bladder and common duct	7 weeks	22·2	43	„	„
22	72	Gall-stones in common duct	9 „	31·7	11	„	„
23	66	Gall-stones; ruptured gall-bladder	6 „	29·0	21	„	„
24	..	Gall-stones; chronic pancreatitis	4 days	12·9	15	„	„
25	57	Gall-stones (also cardiac disease)	13 weeks	31·8	7	Mixed	Radiological.
26	75	Gall-stones in common duct	?	27·5	13·8	Direct	Operation.
27	33	Gall-stone in cystic duct; inflammation of common bile-duct	10 days	20·2	13	„	„
28	44	Gall-stones	?	25·1	5·4	„	Clinical data.

TABLE II.—*Toxic and Infective Jaundice.*

Case.	Age.	Diagnosis.	Duration of jaundice.	Phosphatase units.	Bilirubin. Total units.	Bilirubin. Type of reaction.	Confirmation of diagnosis.
29	52	Catarrhal jaundice	7 days	16·6	55	Direct	Clinical data
30	..	„ „	9 „	13·7	9	„	„ „
31	38	„ „	3 weeks	11·3	48	„	„ „
32	4	„ „	18 days	7·6	48	„	„ „
33	10	„ „	2 „	11·8	21	„	„ „
34	53	„ „	16 „	9·1	9	„	„ „
35	53	„ „	3 weeks	15·0	84	„	„ „
36	30	„ „	10 days	6·8	3·1	„	„ „
37	22	„ „	?	6·4	6·6	Mixed	„ „
38	27	Weil's disease	3 days	9·4	50	Direct	„ „
39	34	„ „	1 day	5·5	52	„	„ „
40	26	„ „	16 days	8·7	23	„	Spirochætes found.
41	51	„ „	14 „	8·2	40	„	Clinical data.
42	52	Weil's disease (fatal)	4 „	8·0	115	„	Post-mortem.
43	21	Infective jaundice, suppurative cholangitis (fatal)	4 „	15·6	100	„	„
44	25	Acute hepatitis, acute broncho-pneumonia (fatal)	7 „	6·4	128	„	„
45	35	Acute hepatitis (acute rheumatism and nephritis)	18 „	18·1	11	„	„
46	18	Ulcerative gastro-enteritis and peritonitis Fatty liver (fatal)	?	10·4	2·2	„	„
47	24	Malaria	1 month	2·8	3	„	Parasites found.
48	26	Mastoiditis, pyæmia, amyloid disease of liver (fatal)	2 months	15·7	12	„	Post-mortem.
49	44	Jaundice following N.A.B. treatment	14 days	21·0	8	„	Clinical data.
50	54	„ „ „ „	4 weeks	16·9	35	„	„ „
			6 „	33·4	12	„	„ „
51	31	„ „ „ „	6 days	14·3	48	„	„ „
52	53	Lupus erythematosus. Treated with gold	?	9·5	8	„	„ „

Cases 38 *to* 42.—The patients were all miners working in pits where numerous cases of jaundice were met with. Cases 38 to 41 all came from the same seam of the same pit, and the finding of the *Leptospira* in one case confirmed the diagnosis for all. Case 42 came from a different pit, where there was also an epidemic.

TABLE III.—*Jaundice in Cardiac Disease with Congestion of the Liver.*

Case.	Age.	Diagnosis.	Phosphatase units.	Bilirubin. Total units.	Bilirubin. Type of reaction.
53	25	Congenital malformation of heart	3·6	4	Indirect.
54	31	Mitral stenosis and regurgitation	6·3	15	„
55	60	Aortic regurgitation	11·2	4	„
56	48	Infective endocarditis	8·6	28	Direct.

TABLE IV.—*Hæmolytic Jaundice.*

Case.	Age.	Diagnosis.	Fragility of corpuscles.	Phosphatase units.	Bilirubin. Total units.	Bilirubin. Type of reaction.
57	16	Splenomegaly and anæmia	Normal	6·9	4	Indirect.
58	27	„ „ „ (fatal)	„	5·9	5	„
59	26	Hæmolytic anæmia	„	5·0	10	„
60	32	Splenomegaly and anæmia	„	5·3	9	„
61	29	Pernicious anæmia	„	4·0	3	„
62	26	„ „	„	7·9	5	„
63	62	„ „	„	5·9	3	„
64	72	„ „	—	4·5	6	„
65	58	„ „	—	2·6	8	„
66	..	Familial acholuric jaundice	Increased	6·8	7	„
67	..	„ „ „	„	6·7	9	„
68	..	„ „ „	„	9·1	10	„
69	36	„ „ „	„	6·7	11	„
70	7	„ „ „	„	13·4	7	„

TABLE V.—*Miscellaneous Cases.*

Case.	Age.	Diagnosis.	Duration of jaundice.	Phosphatase units.	Bilirubin. Total units.	Bilirubin. Type of reaction.
71	64	Cirrhosis of liver	..	7·4	1	Indirect.
72	63	Cirrhosis of liver (following malaria)	..	56·3	Normal	„
73	39	Cholecystitis and cholelithiasis without jaundice	..	5·2	0	Negative.
74	66	? Intrahepatic obstruction to ducts	4 months	46·3	13	Direct.
75	..	Pleural effusion, anæmia, and jaundice during pregnancy	?	32·6	2	„
76	58	Multiple peripheral neuritis	10 weeks	9·3	2·8	„
77	..	Jaundice with greatly enlarged liver (recovered completely)	6 „	21·8	37	„
78	..	? Carcinoma of liver	1 week	38·3	17	„
79	51	? Obstructive jaundice (fatal)	3 weeks	54·4	68	„
80	56	? Subacute hepatitis (fatal)	8 „	10·1	60	„

In the present work, 28 cases of gross mechanical obstruction to the bile-ducts have been examined, and show a range of plasma phosphatase figures from 12·9 to 120 units, the majority of the values being above 20 units (Table I). The cases of toxic or infective jaundice are collected in Table II; the range of phosphatase values in this group is from 2·8 to 33·4 units, but in only 2 out of 22 cases are the figures above 20 units. In hæmolytic jaundice (Table IV) the plasma phosphatase varies from 2·6 to 13·4. The distribution of the data is given in Table VI, which clearly shows the difference between the three types of jaundice. In obstructive jaundice the plasma phosphatase is always

above normal, and usually is very high. In toxic or infective jaundice the plasma phosphatase may be normal or slightly raised, and the range of figures overlaps with that found in the obstructive group. In hæmolytic jaundice the figures are normal or show no significant rise.

TABLE VI.

	Number of observations in which the plasma phosphatase falls in the range:			
	0 to 10 units.	10 to 20 units.	20 to 30 units.	Above 30 units.
Obstructive jaundice	0	5	11	16
Toxic and infective jaundice	12	10	1	1
Hæmolytic jaundice	13	1	0	0

There is no correlation between the intensity of jaundice and the height of the phosphatase figure. Within the obstructive group, a very intense jaundice may be associated with a rise in plasma phosphatase which is only moderate (*e. g.* in Case 17), or a very high phosphatase value may occur in association with a slight degree of jaundice (*e. g.* in Cases 6 and 14).

In toxic or infective jaundice there may be extremely high bilirubin figures, with a direct Van den Bergh reaction, associated with little or no rise in the plasma phosphatase (*e. g.* in Cases 42 and 44).

In the cases of hæmolytic jaundice observed in the course of the present work, there are no instances of very intense jaundice; the bilirubin figures range from 3 to 11 units. The moderate degree of jaundice occurring in this group does not explain the absence of high phosphatase figures. In Table I there are data on 7 cases of obstructive jaundice in which the bilirubin values fall in the same range as those found in the hæmolytic group, and in all these the phosphatase figures are above normal, and in some very high.

In Table III are given the results for 4 cases of jaundice associated with congestion of the liver due to cardiac decompensation. The plasma phosphatase is normal or very slightly raised, and in 3 out of the 4 cases there is an indirect Van den Bergh reaction. Where the Van den Bergh reaction is of the indirect type, the jaundice may be the result either of toxic damage to the liver-cells, or of local blood destruction in the congested liver. In the one case where there is a direct Van den Bergh reaction, the jaundice is probably due to toxic damage to the liver.

In Table V are collected cases which cannot be definitely classified in any of the preceding groups. Cases 71, 72 and 73 represent diseases of the liver and bile-passages without jaundice, though in Case 72 there had been jaundice at an earlier stage, and the plasma phosphatase was high. In the remaining cases the diagnosis is not sufficiently certain to justify their inclusion in the groups to which they are believed to belong.

DISCUSSION.

Bile contains phosphatase, as has been shown by Roberts (1933), and by Greene, Shattuck and Kaplowitz (1934). In the course of the present work, also, the writer has demonstrated phosphatase activity in samples of bile

obtained by drainage after operations on the bile-ducts. It would therefore be expected that occlusion of the ducts would lead to retention of phosphatase, but one cannot say whether this is the only factor which determines high plasma phosphatase values in jaundice. The normal plasma phosphatase is thought to be derived mainly if not entirely from the bones, which contain more phosphatase than any other tissue, but phosphatase is present in many organs, including the liver (Macfarlane, Patterson and Robison, 1934), and the plasma phosphatase may therefore be in part derived from these sources. Bodansky (1934) has shown that the plasma phosphatase may be influenced by diet, and this must indicate an origin of plasma phosphatase from some source other than bone.

Bodansky and Jaffe (1933) have reported plasma phosphatase figures above normal in catarrhal jaundice, and have interpreted this as indicating a hepatic origin of plasma phosphatase. Their method was that of Bodansky (1933). Greene, Shattuck and Kaplowitz (1934), also using Bodansky's method, found high values for plasma phosphatase in catarrhal jaundice, and other types of toxic or infective jaundice, as well as in jaundice due to mechanical obstruction of the ducts. They considered that there was no significant difference between obstructive jaundice and jaundice of the toxic or infective type.

Unfortunately the unit of Bodansky's method cannot be translated into the same terms as the unit used by Roberts or the unit of the method used in the present work, but there is evidently a difference between the results of different authors. Roberts found a perfectly sharp limit between the range of phosphatase figures found in toxic or infective jaundice and the range found in obstructive jaundice; there was no overlapping of the data in the two groups. Greene, Shattuck and Kaplowitz found such a wide overlapping of the data in the different groups that they could make no distinction between toxic jaundice and jaundice due to mechanical obstruction.

The results of the present paper occupy an intermediate position. They show clearly an association of high phosphatase figures with obstructive jaundice, but indicate that in a certain proportion of toxic or infective cases moderate rises, or rarely, marked rises in the plasma phosphatase may occur. The most probable explanation seems to be that there is an obstructive factor present in jaundice of the toxic or infective type, and that the obstructive factor has more effect in some cases than others. If this is so, then there is room for chance to play a part in any short series of observations; different workers may happen to meet a different proportion of toxic cases in which the obstructive factor is pronounced. One would certainly expect some degree of obstruction to the bile capillaries in acute hepatitis; the whole liver is swollen, and the intrahepatic capillaries and canaliculi must surely be compressed. Where there is associated cholangitis, the presence of inflammatory exudate within the ducts may cause obstruction to the outflow of bile. If the rises in plasma phosphatase which are met with in obstructive jaundice, and to a less extent in toxic and infective jaundice, are interpreted as due to varying degrees of biliary obstruction, it is necessary to attempt some explanation of the fact that there is no parallelism between the concentration of phosphatase and of directly-reacting bilirubin in the plasma. Even in jaundice

due to gross mechanical obstruction of the ducts, the concentrations of the two substances vary independently, and in acute hepatitis it is possible to find very intense jaundice with little or no rise in phosphatase.

The concentration of bilirubin and of phosphatase in the plasma in a case of biliary obstruction will depend not only on the degree of obstruction, but also on the rate of production of bilirubin and phosphatase in the body, and on the rate of excretion by channels other than the bile-ducts. Nothing is known of the possible variations in the production of phosphatase in these cases. As regards bilirubin, it is probable that differences in the rate of blood destruction, and therefore of bilirubin formation, occur in different cases, and one would expect an increase in conditions of toxæmia. The effect of increased formation in the presence of obstruction would be an intense jaundice predominantly due to directly-reacting bilirubin, for the greater part of the pigment would be taken up by the liver-cells, transformed to the directly-reacting type, and retained in that form. It is noteworthy that in three fulminant and rapidly fatal cases of acute hepatitis reported in the present series (Cases 42, 43 and 44) the plasma bilirubin was above 100 units—a value very rarely reached even in long-standing jaundice due to mechanical obstruction alone. In such cases it is reasonable to postulate an increased production of bilirubin to account for the intensity of the jaundice. The pigment was almost entirely directly-reacting bilirubin; in Cases 42 and 44, this was confirmed by the absence of any abnormal amount of pigment soluble in chloroform.

An abnormal permeability of the damaged liver-cells, allowing bilirubin to diffuse out readily into the tissue spaces, may also be a factor in causing toxic jaundice.

In considering the plasma phosphatase values, it is necessary to take into account the influence of the duration of obstruction. Roberts has suggested that in long-standing biliary obstruction the plasma phosphatase may steadily accumulate, because it is not excreted to any extend by the kidney. Bilirubin is excreted, and would not be expected to accumulate to the same extent. Case 10 of the present series appears to illustrate such conditions, for the phosphatase increased greatly during ten months, while the bilirubin showed a slight fall. In Case 5, however, there was very little change in three months. Bodansky and Jaffe (1934) have shown that when the common duct is ligatured in a dog, the plasma phosphatase rises rapidly to a very high level. Similar results have been obtained by Armstrong, King and Harris (1934). Possibly a sudden and complete obstruction such as this may cause a more rapid rise than would occur as a result of a gradually progressive obstruction due to a growing tumour.

It is evident from Table I that there is no close correlation between the duration of jaundice and the level of plasma phosphatase. This would not be expected, for the degree of obstruction will vary in the different cases. It is noticeable, however, that very high phosphatase figures are more frequent among the cases of obstruction due to malignant disease, in which obstruction is continuous and progressive, than among cases of obstruction due to gall-stones, in which the obstruction is often incomplete and intermittent. It is of interest, too, to consider those cases in Table I which show a phosphatase

value below 20 units. In Cases 5 and 25 the obstruction had only been present a few days; in Cases 13 and 20 the obstruction was certainly incomplete, and in Case 10 the plasma phosphatase rose later on. The results are, on the whole, consistent with the view that the plasma phosphatase may be only slightly raised when the biliary obstruction is either very recent or incomplete.

In cases of jaundice due to toxic and infective conditions, biliary obstruction is often incomplete, and the patients are often seen shortly after the onset of jaundice. This may be a reason for the finding of plasma phosphatase values within the normal range, or only moderately raised. Roberts states that where toxic jaundice is of long standing, one still sees no appreciable rise in plasma phosphatase. The present work affords only three instances of toxic or infective jaundice lasting more than a month, namely Cases 48, 50 and 80, though in the last case the diagnosis was not fully established. In Cases 48 and 50 there was some rise in plasma phosphatase. In Case 80 there was only an insignificant rise, but in this case bile-pigment was present in the stools, and the incompleteness of the obstruction may account for the absence of greater phosphatase retention.

In the writer's opinion, therefore, the intense jaundice with directly-reacting bilirubin in the plasma, which may occur in acute hepatitis, is probably explained by the association of biliary obstruction with increased production of bilirubin in the body, and the fact that there is usually only a minor degree of phosphatase retention is accounted for by the fact that the biliary obstruction is usually either incomplete or of recent origin. In some cases the biliary obstruction is sufficiently great to cause a rise in plasma phosphatase. It is clearly impossible to determine the effect of these various factors in any one case, but this description makes it possible to interpret the rise in plasma phosphatase as due to biliary obstruction without departing from the accepted theory of the Van den Bergh reaction.

Roberts, on the other hand, tentatively suggested an explanation which involved a fundamental change in the theory of the Van den Bergh reaction. He suggested that directly-reacting bilirubin may be formed in the blood-stream, and that the liver-cells normally may merely transfer it from the blood to the bile. Failure in the excretory function of the cells would then cause an accumulation of directly-reacting bilirubin in the plasma. If this view were correct, it would be difficult to explain the absence of directly-reacting pigment from plasma in normal subjects and in hæmolytic jaundice; for if indirectly-reacting bilirubin can be changed to directly-reacting bilirubin in the circulating blood without the intervention of the liver-cells, there would seem to be no reason why this change should occur only in the hepatic circulation.

From the standpoint of clinical diagnosis the estimation of plasma phosphatase is of limited value. A figure below 10 units (by the method of Jenner and Kay) would be a definite point against the diagnosis of mechanical obstruction to the ducts; a figure above 35 would be in favour of mechanical obstruction; values falling in the range between 10 and 35 units would be consistent with a diagnosis either of mechanical obstruction or of jaundice of the toxic-infective type. Unfortunately, in many of the cases in which an additional diagnostic test would be helpful, the figures fall within this

range. For example, in Cases 5 and 29 jaundice had been present for about a week when the patients were first seen, and the plasma phosphatase figures were 18·7 and 16·6 respectively. The subsequent history showed that one patient had early malignant disease, causing biliary obstruction, and the other catarrhal jaundice. The phosphatase estimation was no help in distinguishing the two conditions at the early stage. Case 13 is also of interest; the patient had been given anti-syphilitic treatment, and subsequently happened to develop a stricture of the common bile-duct. The plasma phosphatase figure (16·7) would have been equally consistent with the diagnosis of acute hepatitis following the treatment. In one instance, Case 79, where the plasma phosphatase figure was 54·4 units, the patient was at first thought to have catarrhal jaundice; he died later, and the clinical diagnosis was probably wrong. There is no instance of toxic or infective jaundice with a plasma phosphatase figure as high as in this case, but if in fact the jaundice in such cases is essentially obstructive, one would hesitate to say that any particular level of plasma phosphatase was inconsistent with the diagnosis of toxic or infective jaundice.

SUMMARY.

Using the method of Jenner and Kay for phosphatase estimation, it has been found that in jaundice due to gross mechanical obstruction of the bile-ducts, the plasma phosphatase is always above normal, and usually is markedly raised. In jaundice due to toxic or infective disease of the liver, the plasma phosphatase is in many cases normal, and in others usually only slightly raised, though occasional high figures occur. In hæmolytic jaundice the plasma phosphatase is normal, or shows no significant increase.

The diagnostic value of the test is limited by the fact that moderate rises in plasma phosphatase may occur both in jaundice due to mechanical obstruction and in toxic or infective jaundice. The estimation of phosphatase may give assistance in diagnosis in some cases.

The figures for phosphatase and directly-reacting bilirubin in the plasma do not run parallel. It may be possible, however, to explain phosphatase retention as due to biliary obstruction without any alteration in the accepted interpretation of the Van den Bergh reaction.

I wish to express my thanks to the members of the honorary medical and surgical staff of the Royal Victoria Infirmary for giving me the opportunity to investigate their cases and to use their clinical notes. I am also much indebted to the medical and surgical registrars of the hospital, to general practitioners, and to the Lady Almoner, for their assistance in following the progress of patients after their discharge from hospital. I wish also to thank my colleagues on the pathological staff for the use of the notes of their post-mortem examinations.

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PHAGOCYTOSIS OF *B. TYPHOSUS* IN RELATION TO ITS ANTIGENIC STRUCTURE AND TO THE ANTIBODY COMPONENTS OF THE SENSITIZING SERUM.

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IT has long been accepted that both cellular and humoral agents play important rôles in the phenomenon of phagocytosis. While definite knowledge is available as to the cellular elements, the nature of the humoral agents which take part in this reaction has remained shrouded under the names of "opsonins" (Wright and Douglas, 1903, 1904) and "bacteriotropins" (Neufeld and Rimpau, 1904, 1905). The relation of these two serum antibodies to each other and to the antibodies known to be responsible for other immunity reactions has been the subject of numerous investigations. Most of these studies, however, were undertaken at a time when it was believed that the bacterial cell took part in immunity reactions as a composite whole, whereas it is now generally recognized that different antigenic components of the bacterial cell and corresponding counterparts in the serum play distinct and separate rôles in immunity and immunity reactions.

Few observers have applied the newer knowledge of the antigenic structure of bacteria to the study of the mechanisms concerned in phagocytosis. Braun and Nodake (1924), working with the O and H + O variants of *B. proteus X*, came to the conclusion that it is the O antibody rather than the H which is the effective agent in the bacteriotropic action of immune serum. On this authority Felix and Robertson (1928), in their scheme of bacterial antibodies, placed the bacteriotropic effect amongst those immunity reactions which do not result from the combination of H antigen and H antibody, but depend on the interaction of the O antigen and its antibody. Hofmeier (1927), and Mudd, Lucke and Strumia (1933), experimenting with *B. typhosus*, came to another conclusion. According to these workers immune sera containing only flagellar

antibodies promote phagocytosis of flagellate bacilli only, while sera containing only somatic antibodies promote phagocytosis of both the flagellated and non-flagellated organisms.

The present investigation was undertaken to test these conflicting statements, and to determine whether any relationship could be established between H and O antibodies and the opsonins of normal sera and bacteriotropins of immune sera. *B. typhosus* was selected as the test organism. The Vi antigen of *B. typhosus* and its corresponding antibody, recently demonstrated by Felix and Pitt (1934*b*), are not considered in the present paper. The effect of the Vi antibody on phagocytosis will be described in a subsequent communication.

TECHNIQUE.

In view of the great technical difficulties known to be involved in experiments on phagocytosis, it is considered desirable to give a detailed account of the technique followed.

(1) *Leucocytes.*—Rabbits' leucocytes were used throughout. The animal was bled from the jugular vein, and approximately 16 c.c. of blood was collected in a tube containing 4 c.c. of a 4 p.c. solution of sodium citrate in fresh normal saline. If clotting took place the specimen was rejected and the animal again bled from the other jugular. It was found that an aqueous solution of sodium citrate did not give satisfactory results, the leucocytes having a tendency to rupture during centrifugalization. The blood-citrate mixture was transferred to four centrifuge tubes and spun for 18–20 minutes. During the first five minutes the speed was maintained at about 500 revolutions per minute to allow the red cells to settle first, and for the next 15 minutes the mixture was spun at 3000 revolutions per minute. The supernatant fluid was then removed and replaced by fresh normal saline. The tubes were gently rotated between the palms of the hands and the same process of centrifuging and washing with saline repeated twice. Finally, the leucocytes, which formed a thick buffy coat on top of the red cells, were removed with the aid of a capillary pipette. About 4 c.c. of thick leucocytic cream could thus be collected. This suspension was made up with normal saline to a total volume of 5·4 c.c., and distributed in quantities of 0·3 c.c. into the tubes containing the other ingredients of the final phagocytic mixtures. It is necessary to emphasize that the leucocyte suspension must be handled very gently. The normal saline used must be fresh, and its reaction not below pH 7·2.

(2) *Bacterial suspensions.*—The strains of *B. typhosus* used in this investigation were those described in previous papers by Felix and Pitt (1934*a*), and Felix, Bhatnagar and Pitt (1934). They were grown on ordinary nutrient agar at 37° C., subcultured daily, and the perfect "smoothness" of the cultures of all the strains was constantly tested very carefully. When a relatively small number of rough organisms was present in a culture offered for phagocytosis, the phagocytic figures increased considerably. Agar slope cultures grown for 20 to 24 hours at 37° C. were suspended in saline, and the dilutions were standardized by opacity to contain 1600×10^6 organisms per c.c. A standard dose of 0·1 c.c. was added to the phagocytic mixtures. The time allowed to elapse between preparing and adding the bacterial suspensions varied only from 15 to 30 minutes.

(3) *Sera.*—Only rabbit sera were used throughout this investigation. When inactivated normal or immune sera were tested they were complemented by the addition of fresh normal rabbit serum. The O and H titres of the sera were determined by agglutination tests with suspensions of live organisms, according to the technique described by Felix and Pitt (1934*a*). Absorbed sera were obtained by treating immune or normal sera with bacilli killed at 60° C. and twice washed with saline in the centrifuge. For the absorption of high-titre immune sera the growth from Roux bottles was used, and an appropriate dilution of the sera was left in contact with the bacilli for 2 hours at 37° C. In most instances this procedure had to be repeated to effect complete absorption of H or O antibodies. When normal serum was absorbed and it was desired to maintain its complement unimpaired, the absorption was carried out in the cold room, as first recommended by Ehrlich and Morgenroth (1899). The serum and the centrifuged washed bacilli were left in the cold room for one hour before absorption, and 3 c.c. of

undiluted normal serum was then absorbed for 2 hours with the growth from 3–6 agar slopes, corresponding to from 120,000 to 240,000 million organisms.

(4) *Method of experiment.*—The total volume of the phagocytic mixture was in each case 0·5 c.c., made up as follows:

(a) *Serum* (undiluted or diluted)	0·1 c.c.	(0·05 c.c. serum and 0·05 c.c. complement, where the latter was added).
(b) *Bacterial suspension.*	0·1 ,,	(live, standard concentration 1600 × 10^6 organisms per c.c.).
(c) *Suspension of leucocytes*	0·3 ,,	
Total	0·5 c.c.	

The tests were carried out at 37° C., the mixtures being agitated all the time. All the ingredients were prepared or kept in the hot room to allow their temperature to rise to 37° C. They were then mixed in the following order—serum, bacteria, complement, leucocytes—and the mixtures were agitated in a shaker for 10 minutes.

(5) *Preparation, fixation and staining of blood-films.*—The slides were thoroughly roasted in the flame to remove all grease, and films were prepared by taking a drop of the phagocytic mixture of such size that it would spread only about three-fourths of the length of the slide and tail off towards the end. The films were immediately dried in front of an electric fan kept in the hot room, then fixed in methyl alcohol for 10 minutes and stained with Giemsa for 20 minutes. The Giemsa solution was freshly prepared on the morning of the test by adding 0·15 c.c. of pure stain to every c.c. of a 1/1000 solution of potassium carbonate. The blood-films with the stain still on them were dipped in a bowl of running water and washed thoroughly, then dried in front of a fan, without any blotting. The number of bacteria ingested by 100 leucocytes was counted. Most of the leucocytes were found at the two margins and towards the end of the blood-film, and no counts were made on those leucocytes which were found lying either in clumps or near an aggregation of bacteria.

SENSITIZATION BY NORMAL SERUM.

As a preliminary to the following experiments the serum from 25 normal rabbits was tested for its content of natural agglutinins for *B. typhosus*. No H agglutinins were found even in a dilution of 1 in 5. For the estimation of O agglutinins the sensitive strain O 901 was used, and the weakest degree of agglutination which could be estimated with the naked eye was taken as the titre. The results of these agglutination tests are summarized in Table I.

TABLE I.—*Natural O Antibodies for* B. typhosus *in 25 Sera of Normal Rabbits.*

Titre.	Number of sera reacting with strain O 901 of *B. typhosus.*
<1 : 10	1
1 : 10	4
1 : 20	7
1 : 50	6
1 : 100	7
Total	25

Table I shows that only in one of the twenty-five sera tested was the titre of the natural O agglutinin less than 1 in 10, whereas the highest titre

observed in this series (a dilution of 1 in 100) was recorded in a considerable proportion of the animals.

Sera with different contents of O antibody were selected from this series of rabbits, and tested for their opsonic power against a number of strains of *B. typhosus*. An illustrative experiment is reproduced in Table II.

TABLE II.—*Phagocytosis and O Agglutinin Titre of Sensitizing Serum.*

Dilution 1 in 5 of normal serum.		Titre of O agglutinin.	Phagocytosis of strain Ty 2.	
			Number of organisms phagocyted by 100 leucocytes.	Cells taking part (p.c.).
No. 1	Fresh	100	115	71
	Inactivated	100	4	4
No. 21	Fresh	50	51	37
	Inactivated	50	3	3
No. 13	Fresh	10	2	2
	Inactivated	10	0	0
No. 15	Fresh	10	0	0
	Inactivated	10	0	0
Saline control			0	0

Note.—The sera were inactivated by heating the undiluted serum at 56° C. for 30 minutes.

Table II shows that the opsonic effects of the sera strictly corresponded with their O agglutinin titres, and that the opsonic activity was lost after heating at 56° C., though the O titres remained unaltered. Similar experiments performed at various times definitely indicated that when a single strain of *B. typhosus* was tested against a number of fresh normal sera, the degree of phagocytosis invariably depended on the titre of the natural O agglutinin contained in the normal serum.

The relationship between phagocytosis and O agglutination was then investigated in another way. It is known from previous work of Felix and Olitzki (1926) that strains of *B. typhosus* which are resistant to the action of the O agglutinin are also resistant to the bactericidal action of serum, and more recently Felix and Pitt (1934*a*) showed that inagglutinable strains are highly virulent, whereas agglutinable strains are of low virulence. Strains of *B. typhosus* of the three types, viz. inagglutinable, agglutinable, and of intermediate agglutinability, were therefore tested in phagocytosis experiments with normal serum. Table III gives a sample experiment.

From the figures recorded in Table III it is quite evident that there is a very close association between phagocytosis and agglutinability by O antibody. The three inagglutinable strains (Ty 2, Giglioli and Watson) are highly resistant to phagocytosis, whereas the three agglutinable strains (Rawlings, H 901 and Ty 8) are highly susceptible. The two strains of intermediate agglutinability also hold an intermediate position with regard to phagocytosis.

When the figures for serum No. 20 (O titre of 1 in 20) are compared

with those for serum No. 1 (O titre of 1 in 100), it is seen that the higher O titre is reflected by the higher degree of phagocytosis of every strain tested. Thus the correlation between phagocytosis and O agglutinin titre already demonstrated in Table II is again emphasized. Table III further shows that by absorbing the two normal sera with *B. typhosus* at 0° C., most of their opsonic activity was removed. Since this treatment is known to remove the O antibody but to leave the whole or part of the complement intact, the failure of the absorbed sera to stimulate phagocytosis is to be attributed to the absence of the O antibody.

The figures recorded in Table III, which indicate phagocytosis in the presence of inactivated and of absorbed serum respectively, call for further comment. It is seen that with the three agglutinable strains there was quite significant

TABLE III.—*Phagocytosis and Resistance to the Action of the O Agglutinin.*

Dilution 1 in 10 of normal serum.		Titre of O agglutinin.	Phagocytosis of strains of *B. typhosus*.							
			Ty 2	Giglioli.	Watson.	Mrs. S.	Ty 1A	Rawlings.	H 901	Ty 8.
No. 20	Fresh	20	8/8	10/10	14/12	20/20	156/64	240/75	540/88	730/98
	Inactivated	20	0	0	0	1	5/5	4/4	8/8	10/8
	Absorbed at 0° C.	0	0	0	0	0	1	3/3	4/4	7/7
No. 1	Fresh	100	88/72	96/80	108/78	132/84	302/82	410/92	610/96	930/100
	Inactivated	100	2/2	1	1	7/7	10/8	24/18	28/24	34/26
	Absorbed at 0° C.	0	0	0	0	1	0	5/5	4/4	7/7
Saline control			0	0	0	0	0	0	1	0
Type of strain			Inagglutinable			Intermediate		Agglutinable		

Note.—(*a*) Sera absorbed with strain O 901 for 2 hours at 0° C.
(*b*) Titre 0 = a negative result in agglutination in dilution 1 in 5.
(*c*) The numerators of the fractions denote the number of organisms phagocyted by 100 leucocytes, the denominators the number of cells taking part per cent.

phagocytosis in the presence of the inactivated Serum No. 1, which in O agglutination reacted to a titre of 1 in 100. The numbers of organisms of the three agglutinable strains that were phagocyted in the presence of this inactivated serum, were considerably higher than those of the three inagglutinable strains when tested against the fresh serum No. 20, which contained O agglutinins only to a titre of 1 in 20. This clearly indicates that the opsonic action of normal serum is not completely annulled when the complement is inactivated by heating the serum at 56° C. for 30 minutes. The natural O antibody alone, unaided by complement, is quite capable of acting as opsonin, though in much lesser degree than it does in the presence of complement. Provided the strains employed are not of the highly resistant, inagglutinable type, sensitization for phagocytosis is demonstrable with heated "opsonin" much in the same way as with heated "bacteriotropin".

For reasons of economy of space the experiments which were devised to ascertain that with the technique employed the sera absorbed at 0° C. had their complement intact are not reproduced. Mixtures of the two differently treated portions of serum, heated or absorbed, respectively, promoted phagocytosis to almost exactly the same degree as the original untreated serum.

These experiments also indicated that the complementing effect of the absorbed serum was very greatly reduced when it was present in the phagocytic mixture in a dilution higher than 1 in 10. Consequently, in the following experiments with immune sera the complement was throughout used in a final dilution of 1 in 10 (*i. e.* 0·05 c.c. of normal serum in the phagocytic mixtures of a total volume of 0·5 c.c.).

SENSITIZATION BY IMMUNE SERA CONTAINING O AND H ANTIBODIES.

Immune sera containing high-titre O and H antibodies were prepared by immunizing rabbits with suspensions from a number of strains of *B. typhosus* which had been killed by heating for one hour at 60° C. or 70° C. Some rabbits were immunized with live suspensions from strains of the avirulent and highly agglutinable type. The sera were kept in the cold room without the addition of any disinfectant, and their complement was inactivated through prolonged storage. When a number of immune sera were tested in phagocytosis experiments, they were all used in the same dilution (or dilutions), and were complemented by the addition of fresh serum from a normal rabbit known to contain natural O antibody of low titre only.

Strains of *B. typhosus* of the inagglutinable type were invariably found to be relatively resistant to phagocytosis in the presence of high-titre immune sera, in a degree similar to that described in the experiments with normal sera. This is illustrated in Table IV.

TABLE IV.—*Sensitization by Immune Sera Containing O and H Antibodies.*

Immune sera in dilution 1 in 500 plus complement (normal serum No. 20 in dilution 1 in 10).	Titre of antibodies.		Phagocytosis of strains.			
	O.	H.	Ty 2.	Giglioli.	Rawlings.	H 901.
71 11	5,000	20,000	32/32	38/34	438/78	610/92
73 111	20,000	5,000	70/64	80/64	750/100	890/100
64 11	20,000	1,000	64/56	68/40	720/96	910/100
69 11	20,000	2,000	58/53	64/52	690/98	870/100
Complement control (normal serum No. 20 in dilution 1 in 10)	20	0	8/8	10/10	256/72	420/84
Saline control			0	0	2/2	1
Type of strain			Inagglutinable		Agglutinable	

Table IV shows that all the four immune sera tested have a marked sensitizing effect on the two inagglutinable strains Ty 2 and Giglioli. This is evident by comparing the figures recorded for the controls (with complement, *i. e.* normal serum alone) with those tabulated for each of the four immune sera. When, however, the degree of phagocytosis attained with the inagglutinable strains is compared with that observed with the agglutinable strain H 901 and Rawlings, it is seen that the latter are phagocyted more actively in the presence of normal serum alone than are the former in the mixtures of the same normal serum plus high-titre immune serum. The relative increase in phagocytosis, caused by the addition of immune serum, is less conspicuous with the agglutinable than with the inagglutinable strains. This is obviously

due to the inability of the leucocytes to take in more than an average of 8 to 10 bacilli per leucocyte. When this limit is reached or approached the counting of the ingested bacilli is also made less accurate.

With regard to the relationship between the degree of phagocytosis and the content of H and O antibodies in the immune serum, the figures in Table IV give quite definite information. With all four strains phagocytosis occurred in the lowest degree with the serum No. 71, 11, which had the highest H titre but the lowest O titre. The sensitizing effect of the immune sera could therefore be attributed only to the action of the O antibody. It was of course, checked by parallel agglutination tests that H antigen was abundant in the suspensions used in the phagocytosis experiments.

Whenever either pure anti-O sera or mixed anti-O + anti-H sera were tested against suspensions of *B. typhosus* which contained both O and H antigens, the sensitizing effects of the sera invariably showed a close correlation to the titres of O antibody. There was no difference whatever in the degree of phagocytosis, whether H agglutinins, even in very great quantities, were present in the sera or not.

The crucial experiments, however, designed to answer the question whether H agglutinins alone, in the absence of O agglutinins, are capable of promoting phagocytosis, were those illustrated by Table V.

TABLE V.—*Sensitization by Absorbed and Unabsorbed Immune Sera.*

Immune sera in dilution 1 in 200 plus complement (normal serum No. 20 in dilution 1 in 10).		Titre of antibodies.		Phagocytosis of strains.	
		O.	H.	Ty 2.	H 901.
No. 72 11	Absorbed with H and O antigen	0	0	10/10	524/92
	Absorbed with O antigen	0	20,000	8/8	540/88
	Unabsorbed	10,000	20,000	114/65	..
No. 71 11	Absorbed with H and O antigen	0	0	8/8	490/86
	Absorbed with O antigen	0	20,000	10/10	520/94
	Unabsorbed	5000	20,000	58/42	..
Complement control (normal serum No. 20 in dilution 1 in 10)		20	0	8/8	516/88
Saline control				0	1

Note.—Titre 0 = a negative result in agglutination in dilution 1 in 5.

Table V shows that the two sera, when deprived, by absorption, of their O antibody content, were deprived at the same time of their capacity to sensitize the bacilli for phagocytosis. The presence in the absorbed sera of H antibody in as high a titre as 1 in 20,000 did not cause any increase in phagocytosis over that due to the fresh normal serum which served as complement. This was true with both the sensitive strain H 901 and the resistant strain Ty 2. The figures indicating phagocytosis of the strain H 901 in the presence of the unabsorbed sera had to be omitted from the table; they could not be ascertained for this sensitive strain with any degree of accuracy, because of the bacteriolytic action of the O antibody, which was brought into play before the organisms were ingested by leucocytes. It will be noted that in this experiment the phagocytic mixtures contained immune serum in the dilution 1 in 200, whereas in the experiment shown in Table IV the immune sera were used in a dilution of 1 in 500.

DISCUSSION.

The experiments described clearly indicate that in normal serum, as well as in immune serum, the O antibody is responsible for the sensitization of *B. typhosus* which renders the bacteria susceptible to phagocytosis. The H antibody, whether present in the phagocytic mixture alone or simultaneously with O antibody, has no appreciable effect.

The " opsonic " effect of normal serum and the " bacteriotropic " effect of immune serum are the results of the combined action of complement and the natural O antibody of normal serum or the immune O antibody of immune serum. There is no essential difference in the mode of action of " opsonins " and " bacteriotropins ". In so far, therefore, as the experiments in this paper which deal with the nature of the sensitizing body in normal serum constitute a fresh contribution to a discussion of thirty years ago in which, among others, Neufeld and Rimpau (1904), Muir and Martin (1906, 1907), Neufeld and Hüne (1907), G. Dean (1905, 1907*a*) and Ledingham (1907) took part, they give strong support to the views of the two last-named that the sensitizing capacity of a normal serum is the resultant of a normal amboceptor acting in conjunction with complement. This normal amboceptor would now be identified with the O antibody.

With immune sera as well as with normal sera the degree of sensitization depends on both the titre of the O antibody and the susceptibility to its action of the particular strain used.

These results are in harmony with those of Braun and Nodake (1924), but traverse the observations published by Hofmeier (1927) and by Mudd, Lucke and Strumia (1933). It is suggested that the discordance is due to the failure of these workers to pay due attention to the sources of error, which are quite numerous in these technically difficult experiments. It has been shown in this paper that the different resistance to the action of O antibody, which is an inherent property of different strains of *B. typhosus*, decidedly influences the degree of phagocytosis. None of the workers mentioned above has paid any attention to this important factor, but it was clearly foreshadowed in certain striking experiments by G. Dean (1907*b*), who based upon it a test for virulence of strain. In Hofmeier's (1927) study the bacterial suspensions employed in phagocytosis tests were derived from cultures grown either on plain agar or on agar containing 0·1 p.c. of phenol, the medium recommended by Braun (1918) for suppressing flagella and H antigen. It is now known from the work of Felix and Pitt (1934*a*) that growth on phenol agar almost entirely suppresses the resistance to O antibody. The experiments of Mudd, Lucke and Stromia (1933) were carried out with suspensions of *B. typhosus*, killed and preserved by the addition of formalin. It is quite obvious that this procedure fundamentally alters one of the most essential conditions under which leucocytes normally deal with living bacteria. There can be little doubt that the very marked spontaneous phagocytosis, which these workers described as one of the special difficulties they encountered, was caused by the use of formalin-killed bacilli. With the technique employed in our experiments spontaneous phagocytosis did not occur even when strains of the highly sensitive type were tested.

From the two papers under discussion it is also evident that the authors did not take the precaution to effect truly complete absorption of the O antibody from the sera which they used for the demonstration of the sensitizing power of H antibody. Our experiments show that when non-resistant strains are employed, even inactivated normal sera, containing only very small quantities of O antibody, are capable of causing significant sensitization. The titre of the O antibody was, in our experiments, always estimated in agglutination tests against live suspensions of the highly sensitive strain O 901. Mudd, Lucke and Strumia (1933), however, used for this purpose killed suspensions of O 901 and of a strain of "S. Stanley"—a procedure well known to result in considerably lower readings of O agglutination. Even with these reagents of inferior sensitiveness residual "O" agglutinins were demonstrated in their absorbed sera. The alleged phagocytosis-promoting effect of H antibodies, observed by Mudd, Lucke and Strumia and stated by them to be "inferior both in completeness and in titre to the agglutination", was therefore most probably due to the residual O agglutinins contained in their incompletely exhausted sera.

It may not be unnecessary again to emphasize the importance in these experiments of assuring perfect smoothness of the cultures used. The presence of rough variants, even in a very small proportion, cause distinct spontaneous phagocytosis and vitiate the results.

SUMMARY.

1. The "opsonic" effect of normal serum is due to the combined action of complement and natural O antibody.

2. The "bacteriotropic" effect of immune serum is due to the combined action of complement and immune O antibody.

3. There is no essential difference between "opsonic" and "bacteriotropic" serum activity; both are demonstrable in the absence of complement, though in considerably lesser degree.

4. The H antibody has no appreciable effect on phagocytosis.

5. Phagocytosis of *B. typhosus* is intimately associated with agglutinability by O antibody. Inagglutinable strains are highly resistant, while agglutinable strains are highly susceptible to phagocytosis.

It is with great pleasure that I express my indebtedness to the Director and governing body of the Lister Institute for the facilities afforded me in this work, and to Dr. Felix, who guided me throughout the course of this investigation.

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THE RELATIVE PATHOGENICITY FOR THE RABBIT OF GRAVIS, MITIS AND INTERMEDIATE STRAINS OF *C. DIPHTHERIÆ*.

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IT was demonstrated by McLeod in 1931 that *C. diphtheriæ* could be divided into three cultural types; these he named gravis, mitis and intermediate respectively. The name "gravis" was chosen because of the association between strains of this type and severe clinical cases of the disease. The present communication describes an effort to determine, by a quantitative method, whether there is any difference in pathogenicity between the three types when the rabbit is used as the experimental animal. It was initiated largely as the result of a chance observation. During experiments with the object of obtaining from rabbits antisera of sufficiently high titre for serological work, an attempt was made to raise the titre by a final intravenous dose of live organisms after a prolonged course of injections with killed cultures. In almost every instance rabbits injected with gravis organisms died, while those injected with mitis organisms usually survived.

Experimental Methods.

With these results in mind, a technique was evolved which was an extension and adaption of that used in obtaining antisera. Briefly it was as follows:

Rabbits weighing 1·5–2 kg. were used, and all inoculations were made intravenously at 5-day intervals in the following doses. (The suspensions were matched in each instance to the standards of Brown's scale.)

2000 million heated to 60° C. for 1 hour.
2000 „ „ „ „
2000 „ „ „ „
2000 „ „ „ „
3000 „ „ „ „
3000 „ „ „ „
5000 „ „ „ „
5000 „ „ „ „
1000 „ alive plus 0·1 c.c. antitoxin (160 units).
2000 „ „ „ „ „
3000 „ „ „ „ „
4000 „ „ „ „ „
5000 „ „ „ „ „
5000 „ „ „ „ „

The antitoxin used throughout was that of Burroughs, Wellcome & Company. Each inoculation was made up to a bulk of 1 c.c. in sterile normal saline. The organisms were grown on 10 p.c. ox serum agar for twenty-four hours, prior to being washed off and injected into the experimental animals. In each group of animals (66 were used in all) equal numbers of mitis, gravis and intermediate strains were used, so that there were 22 strains of each type.

As any complication such as paralysis developed it was at once noted. Upon the death of an animal it was immediately examined, or if the death occurred after 7.30 p.m. it was examined the following morning. All the animals surviving the whole series of injections were killed one week later and examined *post mortem*.

Results.

Relative pathogenicity.—The observation that the intravenous injection of living cultures of *C. diphtheriæ* causes death in rabbits is no new one, although one has been unable to find any reference in the literature to a similar result in animals which had previously received a series of inoculations with killed cultures. Roux and Yersin (1888) found that 1 c.c. of emulsion from serum cultures killed rabbits in sixty hours, and Metin (1898) was also successful. Loeffler (1884), Klein (1889) and Beck (1890), however, introduced large quantities of living culture of *C. diphtheriæ* intravenously into rabbits without positive results.

Table I shows that living cultures of *C. diphtheriæ* killed 51 p.c. of a series of 66 rabbits when inoculated intravenously, and this despite the fact that the animals had previously received a long series of killed cultures in order to

TABLE I.—*Results of Inoculating* 66 *Rabbits with* C. diphtheriæ.

Type.	Number of strains.	Died as result of injections.	Killed after completion of series.	Showing post-mortem fatty degeneration.	Showing no post-mortem change.
Mitis . .	22	7 (32 p.c.)	15 (68 p.c.)	7 (32 p.c.)	15 (68 p.c.)
Intermediate .	22	11 (50 „)	11 (50 „)	11 (50 „)	11 (50 „)
Gravis . .	22	16 (73 „)	6 (27 „)	18 (82 „)	4 (18 „)

develop a basal immunity, and were given antitoxin concurrently with the live cultures. Closer analysis shows that the pathogenicity of the organisms was not uniform over the whole series. The strains whose cultural characters conformed to those of McLeod's mitis type had a mortality of 32 p.c., while that of the gravis type was 73 p.c., and the intermediate type lay between these two extremes.

Table II gives further confirmation of these findings. It shows that of the fatal gravis cases a higher percentage occurred at an early point in the series of inoculations than was the case with the corresponding mitis series. Of 16 deaths due to the gravis strains, 15 (94 p.c.) died before receiving the fourth injection of live organisms (4000 m.), while only 57 p.c. of the 7 deaths due to mitis strains had occurred at this point. Again the intermediate strains occupy an intermediate position—7 (64 p.c.)—at the corresponding point in the series.

The work of Povitsky, Eisner and Jackson (1933) showed that a smaller number of gravis organisms (3000 million) was fatal for guinea-pigs than of mitis organisms (12,000 million). The results in Tables I and II show that with equal quantities of gravis and mitis cultures, the former type also shows the greater pathogenicity for the rabbit. So far as this experiment is concerned, then, the title of gravis adopted by McLeod for certain strains is justified.

TABLE II.—*Number of Animals Dying at Various Points in Series.*

Last inoculation given prior to death of the animal.	Dead. 3000 m.	Alive + 0·1 c.c. (160 units) antitoxin.				
		1000 m.	2000 m.	3000 m.	4000 m.	5000 m.
Mitis	..	..	2	2	1	2
Intermediate	1	..	6	..	2	2
Gravis	..	1	13	1	1	..

Post-mortem Findings.

Whether generally successful in producing fatal results by inoculation of *C. diphtheriæ* into rabbits or not, all observers are more or less agreed upon the post-mortem appearances in fatal cases. Of these the most outstanding are an acute fatty degeneration of the liver and an inflammatory state of the kidneys. The series of experiments on which this communication is based proved no exception to the general finding. Table I shows that the fatal cases exhibited fatty degeneration while the surviving cases generally showed none. Two animals, however, which survived the whole series of inoculations with gravis strains showed similar fatty changes when they were killed and examined.

Briefly enumerated the post-mortem changes in fatal cases were as follows:

Liver.—The organ was enlarged, yellow in colour, friable and soft to the touch. In three cases (8·8 p.c. of all fatal cases in this series) it also showed interlobar fibrosis, which in two cases was very pronounced. Microscopically the changes were those of fatty degeneration of the cells of the inner two-thirds of the lobules. There were fat-droplets, both large and small. No necrosis was visible. There was some round-cell infiltration of the portal tracts.

Kidneys.—These were usually slightly enlarged, and showed fatty degeneration and deepening of the cortex, with hæmorrhagic injection of the medulla. The capsule was not adherent, but it was by no means infrequent to find in these cases instead of fatty degeneration that considerable fibrotic change had occurred in the cortex, which was thinned and showed pitting of the surface due to the fibrosis. In such cases the kidneys were smaller than normal. In two cases abscesses were present in the kidneys and yielded a pure culture of *C. diphtheriæ*, which was in each case similar in type to that with which the animal had been injected, viz. a gravis in one case and an intermediate in the other. Microscopically there was fatty degeneration of the cells of the convoluted tubules with many foci of round-cell infiltration.

Suprarenals.—As a rule no obvious change was found, but occasionally there was slight hæmorrhagic injection.

Diaphragm.—In those cases showing marked fatty degeneration, this extended to the diaphragm, which showed tabby-cat striping.

Heart.—Very frequently the pericardial sac was found to be full of clear fluid, from which, however, one never succeeded in culturing any organisms in the eight cases in which it was attempted. The heart was enlarged when fatty degeneration was present, and in such cases the muscle was soft and pale.

Cultures.

Most observers have found that the organisms die out very rapidly—according to Roux and Yersin (1888) within a few hours of inoculation. Metin (1898) found that the incubated spleen was sterile six hours after the animal had been inoculated intravenously with *C. diphtheriæ*.

In 18 animals (6 from each type) cultures were made from the spleen and heart-blood. Only in 4 cases were positive results obtained—3 intermediate strains and 1 gravis. In the two cases already mentioned, the cultures were obtained from kidney abscesses as well as from the spleen. The other two intermediate strains were obtained from the heart blood and spleen respectively of two animals which died three and four days after the last injection given. In the first case it was obtained three days after 4000 million organisms alive + 0·1 c.c. antitoxin, and in the other, four days after 2000 million organisms alive + 0·1 c.c. antitoxin.

Complications.

The two cases with abscesses have already been mentioned. The only other complication to appear was paralysis of the hind quarters, which affected 7 animals. Four of these had been inoculated with intermediate strains, 2 with mitis, and 1 with gravis.

Discussion.

Leete, McLeod and Morrison (1933) found gravis strains to have a greater pathogenicity for rabbits than had mitis strains. On the other hand, Parish, Whatley and O'Brien (1932) declared that mitis strains were at least *as* virulent for guinea-pigs as were gravis strains. The latter workers, however, used such very massive doses (60,000 million organisms subcutaneously in each guinea-pig) that any difference which might exist would tend to be submerged.

The results recorded in Tables I and II support the view that *C. diphtheriæ* gravis has a greater pathogenicity for rabbits than has the intermediate type, and *C. diphtheriæ* intermediate than has the mitis type. Antitoxin administered in equal amounts did not have the same protective power against gravis and intermediate strains that it had against mitis strains.

The distribution of paralysis among the animals is of interest. Leete, McLeod and Morrison (1933) point out that the highest incidence of paralysis in their clinical cases was in those infected by the intermediate type, and this they attributed to a greater proportion of such cases surviving to show this complication. Such an interpretation finds support in these experiments, for with a higher pathogenicity than with mitis strains and a longer survival period than with gravis strains, it is to be expected that animals inoculated with intermediate strains will show the highest incidence of paralysis.

Summary.

A series of experiments is described wherein it is shown that the gravis type of *C. diphtheriæ* has a greater pathogenicity for rabbits than has the intermediate type, and the latter than has the mitis type.

I wish to acknowledge my indebtedness to Prof. Tulloch for much helpful criticism and advice in carrying out this work, and to Prof. McLeod, who supplied me with twelve of the gravis cultures which were used in this series.

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INTERNAL STRUCTURE OF BACTERIAL CELLS.

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Light-Ground Illumination.

When living cells are examined under the microscope transmitted light is usually employed. The object then appears dark against a light background. By this method a certain amount of intracellular detail may be observed in the larger cells (down to 2μ diameter), but in the smaller bacterial cell, with certain exceptions, no internal structure is visible. This is due to the limit

set by the power of resolution of the optical system, and has been defined by Abbe (1910), after Fraunhofer, as the angular distance of the first-order-maximum (where no group of diffracted rays had penetrated a given slit from the second-order-maximum (group of diffracted rays). At least two maxima following each other must reach the objective to produce an image truly representing the object. The smaller the object or, physically expressed, the narrower the slit, the more the distance between the maxima increases, and, in order to obtain an exact image of the object, it is necessary to approximate them closely. It follows that very small objects will require special methods to obtain this result.

According to Abbe, the maxima may be approximated and the power of resolution consequently increased by—

(1) Reducing the wave-length of the light.

(2) Raising the numerical aperture of the optical system, and consequently the refractive index of the liquid between objective and condenser.

The great disadvantage of the first method is that the object cannot be observed directly, as the eye is insensitive to direct images originated by illumination with ultra-violet light (apart from fluorescence images), and indirect photographic records have to be made. Indirect images originated by fluorescence do not offer any advantage regarding resolution compared with images obtained by illumination with visible light.

The second method has the disadvantage that most micro-organisms do not survive in the fluid of high refractive index required, and especially are killed by monobromnaphthalin, which is required for the objective designed by Abbe to obtain the N.A. of 1·60. This objective, therefore, is of little value in biology.

Dark-Ground Illumination.

Although F. H. Wenham invented his paraboloid in 1850 and J. W. Stephenson his condenser in 1879; it is only during the last twenty years that the method of dark-ground illumination has come into general use. At first, when there was enthusiasm about the easy perceptibility of spirochætes, it was assumed that by this method the power of resolution of the optical system was increased, but later it was thought to be less than with light-ground illumination, due to certain distortions and the peculiar character of the image.

It remained for M. Berek (1926) to show that the power of resolution was equally good with both methods, but that the dark-ground method increased perception owing to the greater contrast between the brightly illuminated object and the dark background. Thus, dark-ground illumination will not enable one to separate two points lying very close to one another any better than light-ground illumination (resolution), but a single point that is invisible by light field, owing to the excess of illumination (irradiation), may be easily observed by the dark-ground method (contrast). Since with dark-ground illumination there is no direct path from the source of light to the eye and only rays diffracted on the object are observed, the greater the intensity of the source of light, the greater will be the contrast between the illuminated object and the dark background, and, therefore, the lower will be the threshold of

perception. In consequence of this increased perception, smaller particles will be rendered visible, but, since the resolving power has not been increased, nothing can be concluded as to their form. Thus, if the length of the diffracting surface is below the limit of resolution of the objective, it will appear as a disc in the microscopical image. If the length of the diffracting surface is above the limit of resolution, the disc will be extended to form a ribbon. If, however, the power of resolution of the optical system is increased by employing one of the methods already enumerated, the dark-ground method offers the most promising method of studying the internal structure of bacteria. All the more so as the ultraviolet microscope designed by A. Koehler (1904) did not justify the great pains taken to develop it, and did not give results which could not have been obtained with ordinary light.

J. E. Barnard (1925) designed his ultraviolet microscope for the use of dark-ground illumination, thus realizing the Abbé-Fraunhofer postulate described above. Barnard's very brilliant results are based on the exact construction of his microscope, and the admirable method of locating and focusing the object with invisible rays.

Intracellular Structure of Bacteria.

Staining methods have demonstrated the presence of structures within the bacterial cell, but since they had never been demonstrated in the living state, they have been regarded as artefacts with a few exceptions, *e. g.* structures in anthrax, diphtheria, tetanus, etc. If a bacterium is observed by the usual dark-ground method, it appears as a double-contoured membrane or rather limiting surface, since it is uncertain whether a bacterium has a membrane in the proper botanical sense, and there is no trace of any intracellular structure. Involution forms are excluded from the present discussion, since they are regarded as abnormal cells.

The following is an account of two methods of demonstrating the intracellular structure of bacteria which appears empty by the ordinary method. Preliminary notes fo these methods have already been published (Eisenberg, 1930, 1932).

(1) A thin film of the bacteria is allowed to dry on a slide 0·9 mm. thick and covered with a drop of oil of high refractive index—ordinary immersion oil is sufficient in most cases—and a coverglass 0·1 mm. thick. Bacteria will remain alive under these conditions for a period varying from some minutes to several weeks, according to the organism employed, and may be regarded as normal during the time of examination.

When such a preparation is observed by dark-ground illumination, one no longer sees the double-contoured limiting surface of the bacterium, but only

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Explanation of Photo-micrographs.

B. anthracis.

Fig. 1.—Immersed in water. Dark-ground condenser N.A. 1·20.
Fig. 2.—Immersed in cedar-wood oil. Dark-ground condenser N.A. 1·20.
Fig. 3.—Immersed in cedar-wood oil. Dark-ground condenser N.A. 1·40.
Fig. 7.—Immersed in water. Slit of azimuth diaphragm parallel to upper part of thread.
Fig. 8.—Immersed in water. Slit of azimuth diaphragm parallel to lower part of thread.
Fig. 9.—Immersed in water. Full illumination.

Merling-Eisenberg.

its internal structure. This appearance is brought about in the following way: When the rays of light reach the surface of the cell they are not diffracted, but, owing to the similarity of the refractive index between the oil and the surface of the cell, they penetrate the surface layer, and are diffracted only by the particles within the cell. Thus, an internal structure is demonstrated in the living cell which bears a relation to that shown in fixed and stained preparations (Figs. 1 and 2). Fig. 1 shows some threads of *B. anthracis* with their usual appearance, Fig. 2 shows the same specimen after the immersion liquid had been carefully changed from water to cedar-wood oil.

In continuing these experiments, it was decided to try a condenser of higher numerical aperture, which would enable a high aperture objective to be used, and thereby still further improve the resolving power of the optical system. A condenser was made by E. Leitz-Wetzlar with a numerical aperture 1·40 and, when this was used in conjunction with an apochromat objective N.A. 1·40 stopped down to 1·37, the results shown in Fig. 3 were obtained. The specimen is the same as in Figs. 1 and 2 illuminated by the condenser N.A. 1·40, shown in Fig. 4. An objective stop of only 1·37 N.A., while illuminating with N.A. 1·40, is only possible by reducing the size of the field. This is done by employing a special eye-piece which possesses two movable slit-diaphragms (Fig. 5), which allow the field to be reduced to the size of the object, thus rendering it possible to open the objective diaphragm to the utmost, just maintaining the dark ground. In the same way, irradiation from unwanted particles is avoided—a very useful result in photo-micrography.

(2) The second method does not require the bacteria to be dried and immersed in oil, but makes it possible to observe them in their natural aqueous media. The method depends on arranging a one-sided oblique illumination by fitting an azimuth diaphragm to the dark-ground condenser (Fig. 6).

The effect produced is as follows: When a bacillus is illuminated from one side, the incident light is diffracted on that surface, and it becomes visible whilst the interior of the cell remains dark. The azimuth diaphragm is now rotated so that the slit is parallel to the long axis of the bacillus. The lateral wall of the bacillus, presenting only a small surface to the incident light, causes little diffraction, and allows the light to penetrate the cell and illuminate any structure within it. By this means it is possible to observe a circulation within the bacteria which appears as a moving veil of a green, yellow and yellowish-red colour. According to Rayleigh (1896), Jaentsch (1914, 1921) and others, the size of the particles may be estimated from the colour of the diffracted light, and it would appear that the size of the particles in the present instance are of the order of 0·04μ in diameter.

Since the intensity of the light diffracted by the particles is extremely slight, it has not yet been possible to record them photographically, but experiments on these lines are being continued.

Figs. 7 and 8 show a chain of *B. anthracis* illuminated in the way described above. As the chain is bent about 90°, one part shows the surface, the other the internal structure in two photographs; the azimuth diaphragm is then rotated 90° and the two parts of the thread exchange their appearance. Fig. 9 shows the appearance of the same thread immersed in water for comparison.

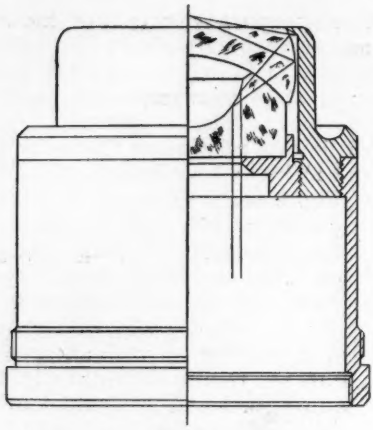

FIG. 4.

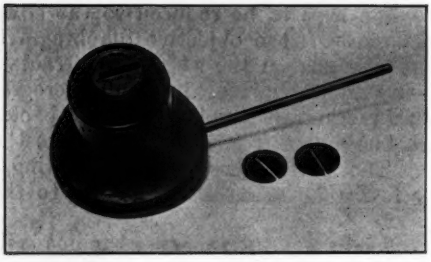

FIG. 5.

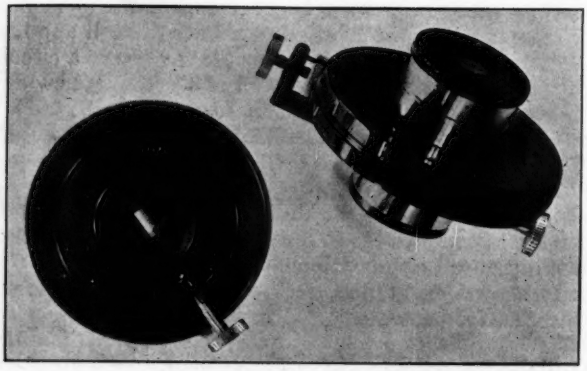

FIG. 6.

Due to unequal transparency of the heat filter used, the two parts of the thread show different intensities.

SUMMARY.

Two methods are described to render visible internal structures in bacterial cells, and illustrations are given.

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THE PROPHYLAXIS OF EXPERIMENTAL *V. SEPTIQUE* INFECTION AND THE PRACTICAL APPLICATION OF ANTIBACTERIAL METHODS.

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IN a recent paper (Henderson, 1934) it was shown that guinea-pigs could be actively immunized against experimental *V. septique* infection with washed and boiled bacillary suspensions. Moderate doses of the vaccine produced only traces of acquired immunity, but intensive and protracted immunization gave 80–100 p.c. protection against the intramuscular inoculation of spore-calcium mixtures.

During the course of experiments designed to demonstrate the mechanism of defence (antibacterial or antitoxic) against such experimental infection, it became clear that antitoxic immunity was also peculiarly difficult to establish

in guinea-pigs. For example, it was shown that 5 c.c. of formolized filtered toxin (given subcutaneously in three doses over a period of 14 days) gave no protection to animals tested 14 days after the last inoculation of vaccine with one certain infecting dose (C.I.D.) of a spore-calcium mixture. With more intensive treatment, similar to that used for antibacterial immunization, the results were still disappointing. In one comparative experiment each of 24 guinea-pigs received 10 c.c. of formolized filtered toxin, and each of 12 guinea-pigs 10 c.c. of " O " antigen (opacity 10 × 10 B. W. & Co., scale). The vaccines were given subcutaneously in six doses over a period of 28 days, and 14 days later all animals were tested intramuscularly with one C.I.D. of a spore-calcium mixture. 46 p.c. of guinea-pigs treated with formolized toxin recovered, while 83 p.c. of animals vaccinated with " O " antigen survived. The mass of bacillary substance used, however, was from 15–20 times the amount recoverable from the whole-culture required in the preparation of the filtered toxin, and in this respect, therefore, the results are not comparable.

The present paper records the results of similar active immunization experiments with rabbits. These tests were undertaken with the object of ascertaining whether the difficulties encountered in immunizing guinea-pigs were peculiar to that species. The success attending the first experiments on immunization with massive doses of bacterial antigen led to the quantitative estimation of the lower limit of bacillary substance necessary to effect protection. Finally, experiments were designed to compare the relative immunizing value of a given volume of formolized whole-culture with (*a*) an equivalent volume of formolized filtered toxin obtained from the same culture and (*b*) the " O " antigen recoverable from a like volume of whole-culture.

In the course of these experiments, sera, collected from the immunized animals, were tested *in vitro*, and subsequently used for passive protection tests in guinea-pigs and mice.

MATERIALS AND METHODS.

The methods of preparation of bacterial vaccines (" O " antigen) and infecting suspensions were similar to those used in previous work (Henderson, 1934). Special reference is made where modifications of technique were introduced.

Formolized whole-culture (" anaculture ") and formolized filtered toxin (" toxoid ") were prepared from 48 hours' growths in 1 p.c. Witte peptone meat broth containing 1 p.c. glucose and a small amount of minced meat. For " anaculture ", part of the whole-culture was siphoned off through a sieve, and for toxoid a further portion was passed through a Berkefeld filter candle. 0·35 p.c. formalin was added to both portions, which were then incubated at 37° C. for 48 hours, and subsequently stored, until required, at ± 1° C.

For experiments on active immunization rabbits of the Agouti variety, weighing 3–4 kg., were selected, except in special instances which are noted. Guinea-pigs weighing 350–400 g. and mice of mixed breed weighing 22–25 g. were used for passive protection experiments.

PRELIMINARY ACTIVE IMMUNIZATION EXPERIMENT.

One group of rabbits received pure toxoid, and another received dense suspensions of boiled bacillary bodies obtained from 10–15 times the volume of whole-culture necessary for the preparation of the toxoid used in the experiment. Each group of animals was subdivided thus: One batch received 10 c.c. of the respective antigen given intravenously in 3 doses over a period of

14 days, and the other received 15 c.c. antigen given in 6 doses (3 subcutaneously, 3 intravenously) over a period of 26 days. The test dose for all animals was given intramuscularly in the leg, and contained 0·5 c.c. of a 10 p.c. $CaCl_2$ solution in distilled water and 0·5 c.c. of the homologous spore suspension in the required dilution. Twenty-one days after the last inoculation of vaccine, half the animals in each batch received one C.I.D. of the spore-calcium mixture, and nine days later the remaining animals, together with survivors of the first test, received a test suspension containing between 10 and 100 C.I.D. The results are recorded in Table I.

TABLE I.—*Preliminary Active Immunization Experiment.*

Antigen for immunization.	Course of immunization.	Number of rabbits.	Preliminary test. Spore-suspension dilution 1 : 1000.		Main test. Spore-suspension dilution 1 : 10.	
			Tested.	Survived.	Tested.	Survived.
"O" antigen	3 inoculations (10 c.c. vaccine)	6	3	3	6	6
	6 inoculations (15 c.c. vaccine)	6	3	3	6	6
Toxoid	3 inoculations (10 c.c. vaccine)	6	3	2	5	1
	6 inoculations (15 c.c. vaccine)	6	3	3	6	4
	Spore-suspension dilution.					
Normal rabbit controls	1 : 10		—	—	5	0
	1 : 100		—		3	0
	1 : 1000		4	0	2	1
	1 : 10,000		2	1	2	1

In respect of acquired antitoxic immunity, these results again demonstrated the difficulty of establishing a solid protection against the severe type of test used. The evidence, however, suggested that a solid antibacterial immunity could be produced with greater facility in rabbits than in guinea-pigs.

As a guide, therefore, to possible field practice, quantitative experiments were commenced to estimate the lower limits of bacillary substance necessary to effect protection. While no minimal effective dose of such a vaccine was determined, it was shown that the bacillary substance obtained from as small a volume as 10 c.c. of whole-culture gave a solid protection, while 10 c.c. of formolized filtered toxin failed to protect any animals subjected to a minimal infecting dose of a spore-calcium mixture.

RELATIVE IMMUNIZING VALUES OF ANACULTURE, TOXOID AND "O" ANTIGEN.

The essential feature of the experiment described below was to compare the degree of immunity that could be established against an intramuscular spore-calcium mixture by the use of a given volume of anaculture, and that which could be produced by (*a*) an equivalent volume of toxoid prepared from the same whole-culture, and (*b*) the "O" antigen recoverable from a like volume of whole-culture.

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Four groups of six animals were used. Rabbits in Group A received 10 c.c. of anaculture intravenously in 3 doses over a period of 13 days. Those in Group B received, in a similar manner, 10 c.c. of toxoid prepared from the same whole-culture. In Group C each rabbit received the "O" antigen which could be recovered from 10 c.c. of the whole-culture. Group D rabbits received the "O" antigen recovered from 10 c.c. of a whole-culture of the same strain of *V. septique* grown in a different medium (1 p.c. glucose tryptic digest broth) yielding a more abundant growth. The "O" antigen in each case was prepared from the centrifuged deposits of known volumes of whole-culture. These deposits were twice washed in large volumes of distilled water, resuspended in distilled water to a known density of not less than opacity 10 × 5 on B. W. & Co.'s scale, and steamed for 2 hours at 100° C. The volume of "O" antigen equivalent to 10 c.c. of whole-culture was calculated, and after the salt concentration was adjusted to 0·85 p.c. with 10 p.c. NaCl, the volume to be used per rabbit was made up to 10 c.c. with physiological salt solution and given intravenously in 3 doses as in Groups A and B. Nineteen days after the last inoculation of vaccines, half the animals in each group received by intramuscular inoculation between 1 and 10 C.I.D. of an homologous spore and calcium mixture. Six days later the remaining untested animals and the survivors of the preliminary test received between 10 and 100 C.I.D. of a similar spore-calcium mixture. The results are given in Table II.

TABLE II.—*Active Immunization: Comparative Value of Anaculture; Toxoid; "O" Antigen.*

Antigen for immunization.	Number of rabbits.	Preliminary test. Spore-suspension dilution 1 : 100.		Main test. Spore-suspension dilution 1 : 10.	
		Tested.	Survived.	Tested.	Survived.
A. Anaculture	5*	3	3	5	5
B. Toxoid	6	3	0	3	0
C. "O" antigen (1)	6	3	3	6	5
D. "O" antigen (2)	6	3	3	6	6
	Spore-suspension dilution.				
Normal rabbit controls	1 : 10	3	0	3	0
	1 : 100	3	0	3	0
	1 : 1000	3	1	3	1
	1 : 10,000	1	1	3	3

* One animal found dead 18 hours after first inoculation of vaccine.

This experiment brings into sharp contrast the solid protection afforded by "O" antigen immunization and the complete failure attending the use of pure toxoid. In Group A (anaculture) it is important to note that one animal (a pregnant female) was found dead 18 hours after the first inoculation of vaccine, and at post-mortem gave evidence of a typical *V. septique* infection. The animal in Group C ("O" antigen) which died of a typical infection 42

hours after testing was of the blue Chinchilla variety, and it is of interest that the " O " agglutinin response in this animal was markedly below that found in all Agouti variety rabbits. The time of death among Group B (toxoid) rabbits was delayed by 12–18 hours over that of normal controls receiving the same test dose. A point of contrast with guinea-pig experiments was the complete absence of external fulminating gangrenes either in controls or in vaccinated animals. Further, the post-mortem picture failed to reveal the extensive blood-stained œdema typical of guinea-pig infection ; as a rule, only traces of œdema surrounded the blackened and necrosed muscle-lesion. The clinical picture was defined by marked local swelling at the site of inoculation, considerable lameness and definite signs of respiratory embarrassment, generally culminating in convulsions and death among controls in from 26–66 hours. Survivors made an uneventful recovery, although signs of lameness were still evident in some animals three weeks after testing.

SEROLOGICAL ANALYSES OF RABBIT IMMUNE SERA.

In the experiments described above all animals were bled before immunization, and again several days before the preliminary test doses were given. The sera were subsequently tested for " O " agglutinin content, antitoxic titre and relative value in passive protection.

" O " agglutinin content of sera.—The technique of agglutination was as previously described (Henderson, 1932*a*), and only homologous " O " antigens steamed for 2 hours at 100° C. were used. The agglutinin titres of individual rabbits receiving " O " antigen or anaculture varied only within narrow limits. In the earlier experiments, where relatively massive doses of " O " antigen were given, a titre of about 1 : 1000 was reached. Where the dosage of vaccine was ten to twenty times less, as in the later experiment, the titre was about 1 : 500, with two exceptions (rabbits other than Agouti) having a titre of about 1 : 200. The " O " titre of sera from animals receiving anaculture appeared to be slightly lower than that of the " O " antigen groups, and it is possible that this result is dependent on the difference in bacterial antigens used respectively for immunization and agglutination (a point later referred to in discussing the results of passive protection experiments). The pure antitoxic sera were devoid of " O " agglutinins, and one animal which had received 20 c.c. of toxoid over a period of 4–5 weeks failed to produce any " O " antibody, which indicates that the results obtained within this group were not affected by antibacterial mechanisms. Table III shows the type of results obtained. The individual sera in each group were pooled. The table also includes for comparative purposes the " O " titre of a rabbit hyper-immunized with pure " O " antigen, and that of various bleedings taken from a horse which had been used by Robertson and Felix (1930) in their experiments on the passive protection of mice with a pure " O " serum. It is interesting to note that this serum had been stored without preservative, at $\pm$ 1° C., since 1929.

Antitoxic content of sera.—The antitoxic titre of the various sera was estimated by the mouse intravenous method, using graded doses of serum against a standard dose of toxin, but owing to the exhaustion of serum supplies it was not possible accurately to titrate any serum to end-point. The results

TABLE III.—*"O" Agglutination Test with Immune Rabbit and Horse-Sera.*

Serum origin.	Serum number.	Agglutinating strain, "KF" (100° C.). Serum dilutions.								
		50.	100.	200.	500.	1000.	2000.	5000.	10,000.	20,000.
Rabbit sera from experiment recorded in Table I	Normal control (pooled)	−	−	−	..	..	..	..	..	..
	"O" antigen Group (3 inoculations)	..	+++	++±	+±	(±)	−	..	..	..
	"O" antigen Group (6 inoculations)	..	+++	++±	++	±	((±))	..	..	..
	Toxoid group (6 inoculations)	−	−	−	..	..	..	..	..	..
Rabbit sera from experiment recorded in Table II	Normal control (pooled)	−	−	−	..	..	..	..	..	..
	Anaculture Group A	..	++	+	((±))	−	−	..	..	..
	Toxoid Group B	−	−	−	..	..	..	..	..	..
	"O" antigen Groups C and D	..	++±	+±	±	−	−	..	..	..
Hyper-immunized rabbit "O" serum	21 (normal control)	−	−	−	..	..	..	..	..	..
	2IV	..	+++	+++	+++	++	+	((±))	−	..
Horse "O" sera (consecutive bleedings)	I (normal control)	−	−	−	..	..	..	..	..	..
	II	..	+++	+±	(±)	−	−	..	..	..
	III	..	+++	+++	+++	++±	++	(±)	−	−
	IV	..	++	++±	+++	+++	+++	+±	±	(±)

+++ to ± = varying degrees of agglutination seen with the naked eye. (±) to ((±)) = traces of agglutination observed with the hand lens.

were based on the provisional standard of Glenny, Llewellyn-Jones and Mason (1931). Sera from rabbits receiving 15 c.c. toxoid (as recorded in Table I) contained between 1 and 5 units of antitoxin per c.c., while sera from other toxoid groups and also from the anaculture group (recorded in Table II) contained between 1/10 and 1 unit per c.c. The sera from rabbits receiving pure " O " antigen contained less than 1/10 unit of antitoxin per c.c.

VALUE OF RABBIT IMMUNE SERA IN PASSIVE PROTECTION.

In passive protection experiments with guinea-pigs and mice the pooled sera from the various groups of rabbits were given intramuscularly 48 hours previous to testing. The test dose consisted of a spore-calcium mixture given intradermally in the shaved abdomen. This method had previously proved to be reliable in guinea-pig experiments, consistently regular deaths occurring among controls ; there was also good evidence to suggest that slight degrees of immunity could be demonstrated by this method, which were not detectable when the intramuscular route was chosen. The results have clearly substantiated the findings of active immunization experiments, and it has been possible to estimate the potency of the various sera in passive protection with reasonable precision.

Guinea-pig tests.—The volume of serum given was generally 2 c.c., and the test dose contained 0·1 c.c. 5 p.c. $CaCl_2$ and 0·1 c.c. spore suspension in the required dilution. All sera with an agglutinin titre of not less than 1 : 500 afforded complete protection against at least 10 C.I.D., while the pure antitoxic sera gave only traces of protection against 1 C.I.D.

Preliminary tests with mice.—As a preliminary to the use of this type of test in mice two problems were investigated. Firstly, it was not clear whether they would respond as guinea-pigs had done to the intradermal method of test. Secondly, Robertson and Felix (1930) had failed to protect mice with rabbit " O " serum, and found intensive and prolonged immunization of a horse necessary for the production of an anti-" O " serum which would protect mice against an intramuscular spore-calcium mixture of *V. septique*. Table IV summarizes the results of an experiment designed to illustrate these points.

Mice in Groups A and B received 1 c.c. normal rabbit serum 48 hours before testing. Group A mice were given an intradermal inoculation containing 0·05 c.c. 5 p.c. $CaCl_2$ and 0·05 c.c. spore suspension in varying decimal dilutions. Group B mice received an intramuscular inoculation containing 0·15 c.c. 5 p.c. $CaCl_2$ and 0·15 c.c. spore suspension in similar decimal dilutions.

Mice in Groups C and D received 1 c.c. of the pooled pure " O " rabbit serum (see Table III). Group C mice were tested intradermally with 1, 10 and 100 C.I.D., and Group D mice were given the intramuscular test doses containing likewise 1, 10 and 100 C.I.D. It is seen that the mice tested by the intradermal route show a greater degree of protection than those tested intramuscularly. The reason for this is not yet clear.

Finally, mice in Groups E and F received respectively varying doses of a hyper-immune rabbit " O " serum (2 IV, Table III), and an anti-" O " horse-serum (III, Table III) with a similar " O " titre. All mice were tested by the intradermal route with 10 or 100 C.I.D., and none succumbed. This indicates

TABLE IV.—*Intradermal* versus *Intramuscular Route of Infection in Mice. Rabbit "O" versus Horse "O" Sera in Mouse Protection Tests.*

Serum group.		Amount of serum given I.M. (c.c.).	Route of infection.	Dilution of test spore-suspension.	Number of mice. Tested.	Survived.
Normal rabbit serum	A	1·00	I.D.	10^{-4}	3	0
				10^{-5}	3	0
				10^{-6}	3	2
	B	1·00	I.M.	10^{-4}	3	0
				10^{-5}	3	0
				10^{-6}	3	0
				10^{-7}	3	2
Rabbit pure "O" serum	C	1·00	I.D.	10^{-3}	3	2
				10^{-4}	3	2
				10^{-5}	3	3
	D	1·00	I.M.	10^{-4}	3	0
				10^{-5}	3	1
				10^{-6}	3	3
Hyper-immune rabbit "O" serum (2 IV)	E	1·00	I.D.	10^{-3}	3	3
				10^{-4}	3	3
		0·25	,,	10^{-3}	3	3
				10^{-4}	3	3
Horse pure "O" serum (III)	F	1·00	,,	10^{-3}	3	3
				10^{-4}	3	3
		0·25	,,	10^{-3}	3	3
				10^{-4}	3	3

the stronger protective power of sera with a higher "O" titre than that given to mice in Groups C and D, but affords no evidence of differences in the protective power of rabbit serum as compared with horse-serum. In view of these results the intradermal method of test was selected for the following experiments.

Comparative protective value for mice of the various rabbit immune sera.—Table V shows the results of one experiment designed to compare the protective value of the pooled sera from the four groups of rabbits noted in Table II. Control mice received 1 c.c. of a serum mixture containing equal volumes of the normal serum from the various groups. It is seen that the lowest certain infecting dilution of the test suspension was 10^{-5}. Mice in each immune serum group, therefore, received respectively 1, 10 and 100 C.I.D. The results are clear. Pure antitoxic serum effected only traces of protection against 1 C.I.D., the pure "O" serum protected against 10 C.I.D., and the mixed antitoxic-anti-"O" serum (Group A anaculture) gave compete protection throughout.

Protective value of anti-anaculture serum (containing "O" antibody + antitoxin).—The properties of this mixed antitoxic-anti-"O" serum were further investigated. In a comparative test with pure "O" serum mice

TABLE V.—*Relative Protective Value of Rabbit Immune Sera (Various).*

Serum group.	Dilution of infecting suspension.	Number of mice. Tested.	Survived.
Groups C and D (" O " antigen)	10^{-3}	3	1
	10^{-4}	3	3
	10^{-5}	3	3
Group A (Anaculture)	10^{-3}	3	3
	10^{-4}	3	3
	10^{-5}	3	3
Group B (Toxoid)	10^{-3}	3	0
	10^{-4}	3	0
	10^{-5}	6	2
Normal rabbit serum	10^{-2}	3	0
	10^{-3}	3	0
	10^{-4}	3	0
	10^{-5}	3	0
	10^{-6}	3	1

received 0·4 c.c. of the respective serum and were tested with 100 C.I.D. of a spore-calcium mixture. The anti-anaculture serum protected four out of five mice, while the pure " O " serum protected only one of five mice.

Since the " O " agglutinin titre of the mixed serum was found to be lower than that of the pure " O " serum (when tested with boiled " O " antigen), the protective power of the former serum was examined after removing, by absorption, the " O " antibody common to both.

Complete absorption of the undiluted serum (9 c.c.) was effected with two applications of the centrifuged deposit from 9 c.c. of a dense suspension of boiled " O " antigen (opacity 10 × 10 on B. W. & Co.'s scale). Each absorption was carried out for 2 hours at 37° C., the first being followed by one of 18 hours at ± 1° C., before removing the organisms by centrifugation. Concurrently, 5 c.c. of the undiluted pure " O " serum was similarly absorbed with the homologous antigen, and 5 c.c. absorbed with a heterologous antigen (*V. septique* strain " Milk ") known to possess a different and highly specific " O " component (Henderson, 1934).

For the in-vivo experiment mice received 1 c.c. of the absorbed sera 48 hours previous to testing, and there were included as controls groups of mice receiving the same sera unabsorbed, the pure antitoxic serum and normal rabbit serum. Table VI summarizes the results.

It is clear that absorption of the pure " O " serum with homologous antigen completely removed the protective antibody, and that absorption with heterologous antigen produced little change. All mice receiving the " O " absorbed anti-anaculture serum survived 1–10 C.I.D., while only one of five mice receiving pure antitoxic serum survived 1 C.I.D. The differences between these last two sera cannot be attributed to wide variation in antitoxic titre, since it was shown by further experiments that doses of pure antitoxin containing at least 5 units fail to give more traces of protection against a similar test dose. It is suggested, therefore, that the explanation of these results may be found in possible differences in the somatic antigen of the unheated culture

TABLE VI.—*Effect of Absorption of "O" Antibody on Protective Value.*

Serum group.	Serum treatment.	Dilution of test spore-suspension.	Number of mice. Tested.	Survived.
Groups C and D ("O" antigen)	Unabsorbed	10^{-3}	4	3
	Absorbed with homologous strain "KF"	10^{-5}	3	0
	Absorbed with heterologous strain "Milk"	10^{-3}	3	1
		10^{-4}	4	4
Group A (Anaculture)	Unabsorbed	10^{-3}	3	3
	Absorbed with homologous strain "KF"	10^{-4}	2	2
		10^{-5}	4	4
Group B (Toxoid)	Untreated	10^{-5}	5	1
Normal rabbit serum	,,	10^{-3}	3	0
		10^{-4}	3	0
		10^{-5}	3	0
		10^{-6}	3	2

(anaculture) and that of the boiled "O" antigen. Should such variation occur, then absorption with boiled bacillary bodies would fail to remove completely the protective antibodies present in the anti-anaculture serum.

DISCUSSION.

The main interest of these results lies in the demonstration of an essentially practical method for the prophylaxis of experimental *V. septique* infections in rabbits; in the illustration afforded of the care necessary in the interpretation of experiments with small animals; and in the development of an in-vivo technique sufficiently reliable and sensitive to demonstrate the protective power of low-titre antibacterial or antitoxic sera prepared against strains of *V. septique*.

The earlier work of Robertson and Felix (1930) and that of the writer (Henderson, 1932*b*, 1933, 1934) on the prophylaxis of experimental "gas-gangrene" infections with pure antibacterial mechanisms had been restricted to experiments with guinea-pigs and mice. The methods employed, necessitating, as they did, intensive and protracted courses of immunization, gave results perhaps more of theoretical than of practical interest. Thus, Craddock and Parish (1931), in their experiments on the serum treatment of experimental *V. septique* infections in mice, commenced to prepare an antibacterial horse-serum, but following several months of immunization, when the protective value was *nil*, concluded that the method was impracticable, particularly in view of the spectacular success attending the use of high-titre pure antitoxic serum. Again, while Weinberg and his collaborators have frequently stressed the possible importance of a mixed antibacterial antitoxic serum in "gas-gangrene" infections, they conclude from recent work (1932) that the preparation of a pure antibacterial serum is not a practical proposition. Coleman

and Gunnison (1931), working with another anaerobe, *Cl. tetani*, prepared antibacterial rabbit and goat sera, but failed to demonstrate protective antibodies against an activated spore infection in guinea-pigs. The writer's experiments (Henderson, 1932*b*) on the active immunization of guinea-pigs against experimental *Cl. chauvoei* infection have been criticized by Roberts (1933), who failed to repeat the work with the same success, and by assumption proclaims the method wholly impracticable for the prophylaxis of naturally occurring *Cl. chauvoei* infections in sheep.

Results presented in this paper suggest an explanation of the findings of these authors, and indicate the care necessary in analysing the results of experiments with one animal species in terms of another. Rabbit experiments, when compared with guinea-pig tests, show that no claim can be made as to the impracticable nature of pure antibacterial prophylaxis until field experiments have been conducted with the animals involved in the naturally occurring disease. Likewise, it would be fallacious to conclude that since toxoid immunization of rabbits is ineffective, field prophylaxis with this antigen need not be considered. Further, the success attending rabbit experiments may, in part, have been dependent on the variety of rabbit selected, since there was some indication that " O " agglutinin and active immunity response was lower in varieties other than Agouti.

In passive protection experiments with mice, the calcium chloride technique, though acknowledged to be the only accurate procedure, is particularly severe when applied by the intramuscular route ; there is evidence also for the belief that in previous work this test was insufficiently controlled in so far as concerned the accurate determination of the minimal certain infecting dose. In the present work a comparison of the intra-muscular and intradermal routes of infection has shown the latter to be more sensitive for the estimation of protective values of low-titre antibacterial and antitoxic sera. By this means it has been shown that prolonged courses of immunization for rabbits and horses are not essential for the production of demonstrable protective antibody, either antitoxic or antibacterial. It has also become clear that there is no essential difference in the protective value of antibacterial rabbit or horse-sera, as had previously been suggested. From the examination also of a mixed antibacterial antitoxic rabbit serum, prepared by the use of formolized but unheated whole-culture, there is evidence to suggest that bacilli used as somatic antigen in the unheated state may be antigenically different from the " O " antigen of boiled suspensions, and that this difference results in the production of antibacterial sera of higher protective value.

A problem which arose throughout the course of this work concerned the relative " invasiveness " of various strains of *V. septique* from the same " O " groups (Henderson, 1934). For the preparation of toxoid and anaculture a strain (" KF ") had been selected because of its high toxigenicity. In preliminary tests, however, it was evident that the " invasive " power of this strain was erratic ; only with difficulty could regular infection and death be established, particularly in rabbits. On the other hand, spore-suspensions prepared from the classical strain " Pasteur III ", with identical " O " antigen, gave constant results in this respect. This latter strain was used in some instances in both active and passive protection tests, and antibacterial defence

was equally effective against it and the strain "KF". These variations in relative "invasiveness" and toxigenicity, which have been noted by other workers, may have a practical significance in field prophylaxis. Thus it may be, that where the "invasive" mechanism is dominant, antibacterial defence is of first importance, while where "invasion" is low or *nil* (as in certain *Cl. œdematiens* infections, or in the group of entero-toxæmias), antitoxic immunity takes the first line of defence. Clearly, however, antibacterial defence in the prophylaxis of "gas-gangrene" infections must be reckoned an important factor.

SUMMARY.

1. Rabbits immunized with the "O" antigen recovered from 10 c.c. of a culture of *V. septique* are protected against multiple C.I.D. of $CaCl_2$-activated spore-suspensions. The strains used respectively for vaccination and testing must possess identical "O" antigens.

2. Rabbits immunized with 10 c.c. of a formolized filtrate from the same culture fail to develop an antitoxic immunity sufficient to protect against one C.I.D. of a similar test suspension.

3. 10 c.c. of the same whole-culture, to which formalin is added, produces in rabbits an active immunity against multiple C.I.D. of activated spores.

4. The intradermal route of infection is satisfactory for the titration of activated spore-suspensions of *V. septique*. This test is sufficiently sensitive to demonstrate protective antibody in low-titre antibacterial sera.

5. Pure antibacterial sera, from rabbits shown to be actively immune against spore-calcium infection and with an "O" agglutinin titre of about 1 : 500, protect mice against 1 to 10 C.I.D. of activated spores.

6. With the mouse intradermal test there is no observable difference in the protective values of rabbit and horse "O" sera possessing similar agglutinin titres.

7. The antibody produced in rabbits in response to inoculation with formolized but unheated bacilli possesses a higher protective value than that of the "O" antibody which develops in response to the inoculation of boiled bacillary bodies. This higher value in protection is independent of antitoxic action.

8. The bearing of these results on the prophylaxis of experimental "gas-gangrene" infections is discussed.

I wish to express my sincere thanks to Mr. Dalling of the Wellcome Physiological Research Laboratories, who kindly undertook the preparation of the formolized whole-culture and formolized filtered toxin used in these experiments. I am indebted to him also for the supply of standard antitoxic sera.

To Dr. Robertson and Dr. Felix I convey my thanks for their continued interest and help in this work. I am indebted to them for the supply of antibacterial horse-serum used in the comparative experiments.

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INACTIVATION OF THE "H" ANTIGEN BY DILUTE MINERAL ACID.

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DURING the course of a study of the phenomenon of agglutination, it was noticed, in the case of motile bacteria, that the functions of antibody-absorption and agglutination of the "H" antigens were destroyed by the action of relatively weak concentrations of hydrochloric acid, which left the corresponding functions of the associated "O" antigens unimpaired. For example, in a suspension of *Bact. typhosum* of approximately 750 million organisms to 1·0 c.c. the "H" antigens were rendered inagglutinable in less than one minute, at 50° C., by *M*/1280 hydrochloric acid. The method used in this experiment was to take equal volumes of a suspension of *Bact. typhosum* of 1500 millions to 1·0 c.c. (reaction pH 6·9), *M*/640 hydrochloric acid and a neutralizing concentration of sodium hydrate, and heat them to 50° C. on the water-bath; the acid was then mixed with the suspension and the mixture was neutralized one minute later by adding the alkali. The final mixture, when tested with a *Bact. typhosum* agglutinating serum, gave no "H" antigen-antibody reaction, but the "O" reactions were unimpaired.

Factors Influencing Acid Inactivation of the "H" Antigens.

The minimum effective concentration of the acid is determined by the density of the bacterial suspension acted upon, the duration of action and the temperature. The effects of these three factors are well illustrated in Table I, which shows, over a range of convenient densities, that the minimum inactivating concentration of the acid varies directly with the number of organisms. The velocity of inactivation increases with the temperature; for example, *M*/1280 HCl inactivated the "H" antigens of a suspension of *Bact. typhosum*, 750 millions in 1·0 c.c., in less than 1 minute at 50° C.; but at 18° C. this effect was not attained in 2 hours. For the same *ratio* of bacteria to acid, the temperature being constant, the time necessary for inactivation varies

TABLE I.—*Inactivation of the "H" Antigens of a Suspension of* Bact. typhosum *by Hydrochloric Acid.*

Millions of organisms in 1·0 c.c. of final mixture.	Final concentrations of HCl.								
	M/320.	*M*/480.	*M*/640.	*M*/960.	*M*/1280.	*M*/1920.	*M*/2560.	*M*/3840.	*M*/7680.
Section A:									
3200	++	+++	+s	−	−	−	−	−	−
1600	++	++	+++	+++	−	−	−	−	−
800	++	++	++	+++	+++	+	−	−	−
400	+s	+s	+s	+s	+s	++	−	−	−
200	+s	+s	+s	+s	+s	+s	++	+	−
100	tr.	tr.	tr.	tr.	tr.	tr.	tr.	+	−
Section B:									
3200	−	−	++	++	++	++	++	++	++
1600	−	−	−	++	+++	+++	+++	+++	+++
800	−	−	−	−	−	++	+++	+++	+++
400	−	−	−	−	−	−	++	+++	+++
200	−	−	−	−	−	−	−	++	+++
100	−	−	−	−	−	−	−	tr.	++
Section C:									
3200	−	−	+	++	++	++	++	++	++
1600	−	−	−	−	+++	+++	+++	+++	+++
800	−	−	−	−	−	+++	+++	+++	+++
400	−	−	−	−	−	−	tr.	+++	+++
200	−	−	−	−	−	−	−	+++	+++
100	−	−	−	−	−	−	−	tr.	++
Section D:									
3200	−	−	+	++	++	++	++	++	++
1600	−	−	−	−	+++	+++	+++	+++	+++
800	−	−	−	−	−	−	+++	+++	+++
400	−	−	−	−	−	−	−	+++	+++
200	−	−	−	−	−	−	−	−	+++
100	−	−	−	−	−	−	−	−	+
Section E:									
3200	−	++	++	++	++	++	++	++	++
1600	−	−	+	++	+++	+++	+++	+++	+++
800	−	−	−	++	++	+++	+++	+++	+++
400	−	−	−	+s	++	++	+++	+++	+++
200	−	−	−	tr.	+	+	+++	+++	+++
100	−	−	−	−	+	+	++	+++	+++
Section F:									
3200	−	−	++	++	++	++	++	++	++
1600	−	−	−	+++	+++	+++	+++	+++	+++
800	−	−	−	−	−	+++	+++	+++	+++
400	−	−	−	−	−	−	+++	+++	+++
200	−	−	−	−	−	−	−	+++	+++
100	−	−	−	−	−	−	−	+	+++

Section A: "Acid agglutination" read after 18 hours at 45° C.; similar results were given by incubation at 18° C.

Section B: The suspensions were incubated in the acid dilutions for 1·75 minutes at 50° C., then neutralized with NaOH and tested for "H" agglutination with a *Bact.* "Stanley" serum.

Sections C and D are similar to B, but the times of incubation in acid were 1 hour and 20 hours respectively.

Sections E and F are similar to B, but the incubation in acid was at 18° C., and the times were 15 minutes and 20 hours respectively.

Except in Section A:

+++ = complete "H" agglutination.
+s = slight "H" agglutination.
tr. = trace of "H" agglutination.
− = inactivation of "H" antigen.

inversely with the number of bacteria—that is, with the degree of dilution of the bacteria-acid mixture.

In section A of Table I it will be noticed that the range of direct acid agglutination corresponds closely with that of inactivation of the "H" antigens as shown in sections B, C, D, and F, but it should be mentioned that acid agglutination does not necessarily precede inactivation of the "H" antigens, and, on the other hand, good acid agglutination at the so-called iso-electric point may not preclude a subsequent "H" serum agglutination.

Comparing the results shown in sections B, C and D, it will be seen that the maximum inactivating effect, at 50° C., is attained in the majority of cases within 1·75 minutes; individual tests showed that the minimum time is usually less than 30 seconds. Sections E and F show that when inactivation takes place at room temperature a much longer period of incubation in acid is necessary, or, if inactivation is to be effected within 15 minutes, a higher ratio of acid to bacteria must be used. In order to get a closer approximation to the minimum time necessary for inactivation at the different densities of bacterial suspension, by the minimum effective concentrations of acid, individual tests were set up, in which the acid was neutralized at intervals between 30 seconds and 2 hours, and after 18 to 24 hours. The results of these tests, which were incubated at 50° C. and at 17° to 18° C., are given in Table II.

TABLE II.—*Time Necessary for Inactivation of the "H" Antigens of a Suspension of* Bact. typhosum *by Hydrochloric Acid.*

	Millions of bacilli in 1·0 c.c.	Concentrations of HCl.	Time necessary for inactivation.
Incubation at 50° C.	3200	*M*/320	Within 30 seconds.
	3200	*M*/480	,, 1 minute, not in 30 seconds.
	1600	*M*/640	,, 30 seconds.
	1600	*M*/960	,, 4 minutes, not in 3 minutes.
	800	*M*/1280	,, 1 minute, not in 30 seconds.
	800	*M*/1920	,, 2 hours, not in 1 hour.
	400	*M*/2560	In about 1 hour.
	400	*M*/3840	Not in 24 hours.
Incubation at 17° C. to 18° C.	3200	*M*/320	Within 15 minutes.
	3200	*M*/480	,, 18 hours, not in 2 hours.
	1600	*M*/640	,, 1 hour, not in 30 minutes.
	1600	*M*/960	Not in 24 hours.
	800	*M*/960	Within 2 hours, not in 1 hour.
	800	*M*/1280	,, 18 hours, not in 2 hours.
	400	*M*/960	,, 30 minutes.
	400	*M*/1280	,, 2 hours, not in 1 hour.
	400	*M*/1920	,, 18 hours, not in 2 hours.
	400	*M*/2560	Not in 24 hours.

From Table II it will be seen that, for a constant ratio of bacteria to acid, the time necessary for inactivation increases with the reduction in the number of bacteria—that is, with dilution of the bacteria-acid mixture. For example,

in the suspension of 3200 millions of organisms in 1·0 c.c. of *M*/480 HCl, the "H" antigens were inactivated, at 50° C., in less than 1 minute; but, at the same ratio of bacteria to acid, the suspension of 1600 millions of organisms in 1·0 c.c. of *M*/960 acid required nearly 4 minutes; that of 800 millions of organisms in 1·0 c.c. of *M*/1920 acid required between 1 and 2 hours; and with the suspension of 400 millions of organisms in 1·0 c.c. of *M*/3840 acid inactivation did not occur in 24 hours.

Other factors considered.—Amongst the three species of motile bacteria used—*Bact. typhosum*, *Bact. enteritidis* and *Bact.* "*Stanley*"—no difference in the susceptibility of the "H" antigens to inactivation by acid was noticed.

The presence of sodium chloride was not necessary for inactivation. Fixation by formalin was found, on one occasion, to render the "H" antigen of a culture of *Bact. typhosum* slightly more resistant to acid inactivation than the unfixed control. This resistance, measured by a slight increase in the time or in the concentration of acid necessary for inactivation, varied in degree directly with the concentration of formalin used for fixation. The observation, however, was not confirmed in subsequent tests, and, in fact, it was found advantageous to employ formalin for killing and preserving cultures to be used for acid inactivation.

Effect of Acid on the "O" Antigens.

Inactivation of the "H" antigens usually leads to a marked improvement in the quality of the "O" agglutination, as shown by a more rapid and more complete reaction and, sometimes, extension of the end-point serum titre. This improvement, most evident in suspensions which, before inactivation of their "H" antigens, had given good "H" agglutination and rather poor "O" agglutination, seems to be due to removal of the opposition to "O" agglutination offered by the unchanged and unsensitized "H" antigens.

The concentration of hydrochloric acid (usually about *M*/640), employed for inactivation of the "H" antigens, caused no apparent injury to the "O" antigens, in the time and at the temperature used; but excessively high concentrations of acid, *used at a temperature of 50° C.*, will affect them. In moderate excess the acid may cause some reduction of the agglutinability of the "O" antigens, as shown by a lessening of the velocity of agglutination and a slight shortening of the serum end-point titre; but with great excess, such as *M*/40 acid, the "O" antigens become hypersensitive to serum agglutination, and the end-point titre and the velocity of agglutination are raised. For example, a culture of *Bact. dysenteriæ* Flexner Z was treated for one hour at 50° C. with a series of rising concentrations of HCl—*M*/1280, *M*/640, *M*/320, *M*/160, *M*/80, *M*/40 and *M*/20. The seven suspensions were then neutralized, and tested with the homologous serum for changes in the velocity of agglutination and in the end-point serum titre. As shown by comparison with the result of a control test, treatment with *M*/1280 acid had no effect on the velocity of the subsequent serum agglutination, but there was a slight, progressive fall in the velocity of the reaction following treatment in the acid concentrations from *M*/640 to *M*/160, then a fairly sharp rise, culminating with the suspension treated in *M*/20 acid. The effect on the serum end-titre readings was as

follows : Between the suspension treated in *M*/1280 acid and that treated in *M*/40 acid there was a fall in the end-point titre only from 1/12,800 to 1/9600, but with the suspension treated in *M*/20 acid, the end-point titre was raised well above 1/25,600. Tests of a similar kind on the " O " antigens of *Bact. typhosum* and *Bact. enteritidis* confirmed the findings in the case of *Bact. dysenteriæ*.

Effects of Acid on Other Functions of the Antigens.

Effect on specific absorption of immune body.—Destruction of the agglutinability of the " H " antigens by acid is associated with a corresponding loss of the property of absorbing " H " agglutinin, but the " O " antigens which have preserved their agglutinability also retain, in full, their specific absorbing power.

Effect on agglutinogenesis.—Throughout this paper the expression " inactivation of the 'H' antigens " should be taken to apply only to the functions of agglutination and absorption of agglutinin.
bility and absorbing power, the property of agglutinogenesis may be preserved, showing that the " H " antigens have not been destroyed by the acid.

Four tests of agglutinogenesis were made with acid-inactivated suspensions ; in all cases " H " antibodies were formed ; in two the " H " antibody response was in excess of, and in two less than, that provoked by the control inoculation. Cultures of *Bact. typhosum*, *Bact. enteritidis* and *Bact.* " *Stanley* ", inactivated in a slight excess of acid at 50° C. for 5 minutes, were tested and proved inagglutinable and devoid of absorbing power. These three inactivated suspensions and three corresponding control suspensions were killed by heating to 57° C. for 30 minutes, and inoculated intravenously into rabbits in two doses of 500,000,000 and 1,000,000,000 organisms with an interval of 7 days. Serum was collected 8 days after the second inoculation, and the following end-titres of " H " agglutination were recorded : *Bact. typhosum* inactivated 1/41,000, control 1/10,240 ; *Bact.* " *Stanley* " inactivated 1/1280, control 1/5120 ; *Bact. enteritidis* inactivated 1/160, control 1/20,000. In a previous test with the same strain of *Bact. enteritidis* the inactivated suspension provoked a slightly greater " H " antibody response than the control. The " O " antibody responses to test and control inoculations were approximately equal.

Effect of Acid on the Viability of the Organisms.

Exposure of a living culture to acid, in the concentrations used to inactivate the " H " antigens, reduces the number of surviving organisms, as compared with a control suspension at pH 6·9, maintained at a similar temperature. At the minimum inactivating concentration (M.I.C.) of acid, and the shortest time, the reduction of the number of viable organisms may be very slight, especially when inactivation is effected at room temperature, but when the acid is used in excess of the M.I.C. its lethal effect becomes very marked ; so that with twice the M.I.C. of acid, at 50° C., the surviving organisms number only about 40 to 20 p.c. of the control reading, and at three times the M.I.C. the survivors may number only 10 p.c. after 3 minutes at 50° C., or 15 p.c. after one hour at 18° C.

Sterilization and Preservation of the Inactivated Suspensions.

Much of the work on which this report is based was done with living cultures, but formalin-killed suspensions were also used. Formalin, in sufficient strength to sterilize the culture, may be used either before or after acid inactivation, without causing immediate harm to the " O " antigens; but formolized, inactivated suspensions stored at room temperature for three weeks (but not when stored in the ice-chest) showed a very slight weakening of agglutination at the end-point titre. The observation was not made over a longer period.

Heat sterilization was harmless when used *after* acid inactivation, but it should not be employed *before* exposure to the acid. A suspension of *Bact. enteritidis* heated to 57° C. for 1 hour before acid inactivation showed, in the subsequent serum agglutination, some reduction in " O " agglutinability, and a culture of *Bact. typhosum* heated to 62° C. for 30 minutes before acid inactivation was found to have lost, almost completely, its " O " agglutinability. As a non-flagellated species was not affected in this way, it may be assumed that direct injury is not inflicted on the " O " antigens, but, possibly, they are partly occluded by the altered " H " antigens.

SUMMARY AND CONCLUSION.

The agglutinability and antibody-absorbing properties of the " H " antigens of flagellated bacteria are readily destroyed by exposing the organisms to a suitable, weak, concentration of mineral acid; the appropriate acid concentration for a particular bacterial suspension can be determined by titrating a constant volume of the suspension against a series of falling concentrations of the acid and, after a period, neutralizing the mixtures with sodium hydrate solution and testing them with an " H " agglutinating serum to find the limit of acid inactivation of the " H " antigens. Inactivation is effected very rapidly at 50° C. and less rapidly at 18° C., but, although the weak concentrations of acid used cause no apparent damage to the " O " antigens in a short time at 50° C., it may be advisable to use the lower temperature.

No attempt has been made to compare this technique with other methods used for inactivating the " H " antigens, but it can be commended for its rapidity and simplicity; it does not involve washing or centrifuging the suspension, and it seems to cause little or no damage to the " O " antigens.

Postscript.—In explanation of the rather anomalous finding that the property of agglutinogenesis of the " H " antigens may persist after the loss of their antibody-absorbing power, it is suggested that a small residue of unaltered " H " antigen, undetectable by agglutination or antibody-absorption tests, yet capable of evoking a good " H " antibody response in the rabbit, may remain in the suspension after treatment with acid; especially if the suspension used is dense. Topley, (1930, *J. Path. Bact.*, **33**, 339) has shown that a single intravenous injection of 100,000 organisms per kilogramme of body-weight may evoke a fair " H " antibody response in the rabbit; this dose would represent about 0·01 p.c. of the doses of inactivated suspensions used in the agglutinogenesis tests mentioned above.

The results of experiments made since this paper was submitted for publication give support to this explanation, and it seems probable that the acid-inactivated " H " antigen has no agglutinogenic properties. The *total* removal of " H " agglutinogenesis in any suspension, by acid treatment, is difficult to achieve, but the property may very easily be reduced to such a small degree that successive inoculations of the rabbit are necessary to show its presence.

OBSERVATIONS ON THE LIFE-CYCLE OF THE PLEURO-PNEUMONIA VIRUS.

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THE morphology of the virus of pleuropneumonia has been studied very extensively, owing to the fact that it can be readily cultivated in ordinary media. It is unnecessary to refer to previous work in detail, since the literature has recently been reviewed by Ledingham (1933) and Klieneberger (1934). In this study of the subject a method is described by which the living virus can be seen with ordinary light, and continuous observations of growing cultures of the micro-organism have been made, extending over periods for as long as 21 days. It has thus been possible to observe further details of the mode of growth and of the morphology.

PROCEDURE OF EXAMINATION.

Surface colonies of the pleuropneumonia virus were grown on serum agar in Petri dishes and examined by incident light by means of the Ultropak, with an initial magnification of 1125 diameters. Fig. 1 shows the appearance of a colony by this method. (Negative enlarged × 4, total magnification × 4500.) Spheres can be distinguished, but no detail is visible, since it is characteristic of pleuropneumonia to grow just beneath the surface of this medium.

Much greater detail can be observed by means of dark-ground illumination from below, but, for this purpose, the thickness of the preparation must not exceed 0·1 mm. (Fig. 2). Thin preparations were made as follows: A small piece of agar bearing the colonies was cut from the dish, put between a slide and coverslip, and gently compressed until the required thickness was obtained, when the edges were sealed with melted paraffin wax. Ordinary sterile precautions were, of course, observed, and the glassware was absolutely clean.

These thin cultures were kept continuously on the warm stage of the microscope at 37° C., and were observed at suitable intervals for periods up to three weeks. All the different stages described below were seen in these growing cultures, and although different stages in the life-cycle may be seen in the same culture, prolonged observation of one colony has demonstrated the chain of events in the development of the organism described below.

(1) The most primitive stage seems to be that of the elementary body, the diameter of which can only be estimated very roughly, due to the fact that it appears as a diffraction disc with no sharp contour. The average diameter is approximately 0·1μ, varying by ± 0·02μ. As free elementary bodies are

in very fast Brownian movement, no attempts to obtain photo-micrographs have so far been more than partially successful (Fig. 3). Only when growing in chains did they give sufficient light to be well recorded. Such chains can be seen in Figs. 6 and 7.

(2) The living elementary body develops into an elementary disc, which has hitherto been described as a sphere. Elementary bodies and discs of various sizes are shown in Figs. 3 and 4. Fig. 8 shows larger discs which have grown out of a colony, which is seen as a white mass in a grey background of agar. Fig. 9 shows a chain of elementary discs which has grown from a basal disc of larger diameter. This chain is twisted in the middle, thus showing that the organisms at this stage are really flat discs and not spheres.

(3) After some time the disc, which has usually developed some peripheral elementary bodies (Figs. 4 and 5), grows into a sphere and develops an internal organization; this stage represents a complete virus colony (Fig. 10).

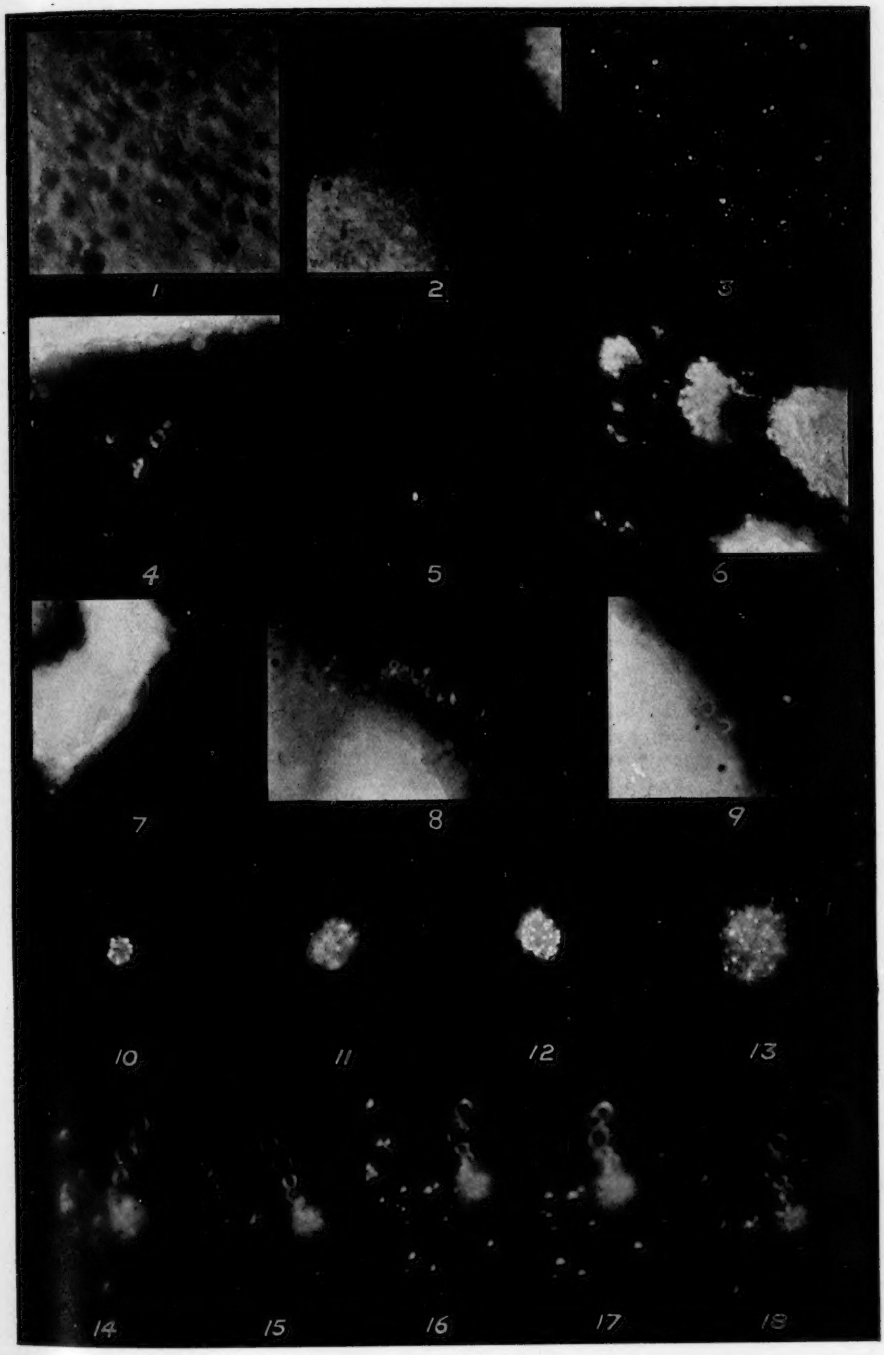
(4) The internal organization now becomes visible as elementary bodies within the sphere, and elementary discs appear outside the sphere. This is shown in Figs. 11 and 12.

(5) The sphere now breaks up, setting free the elementary bodies and discs (Fig. 13), which then show very fast Brownian movement. Before the disintegration of the sphere, the discs may grow out in chains.

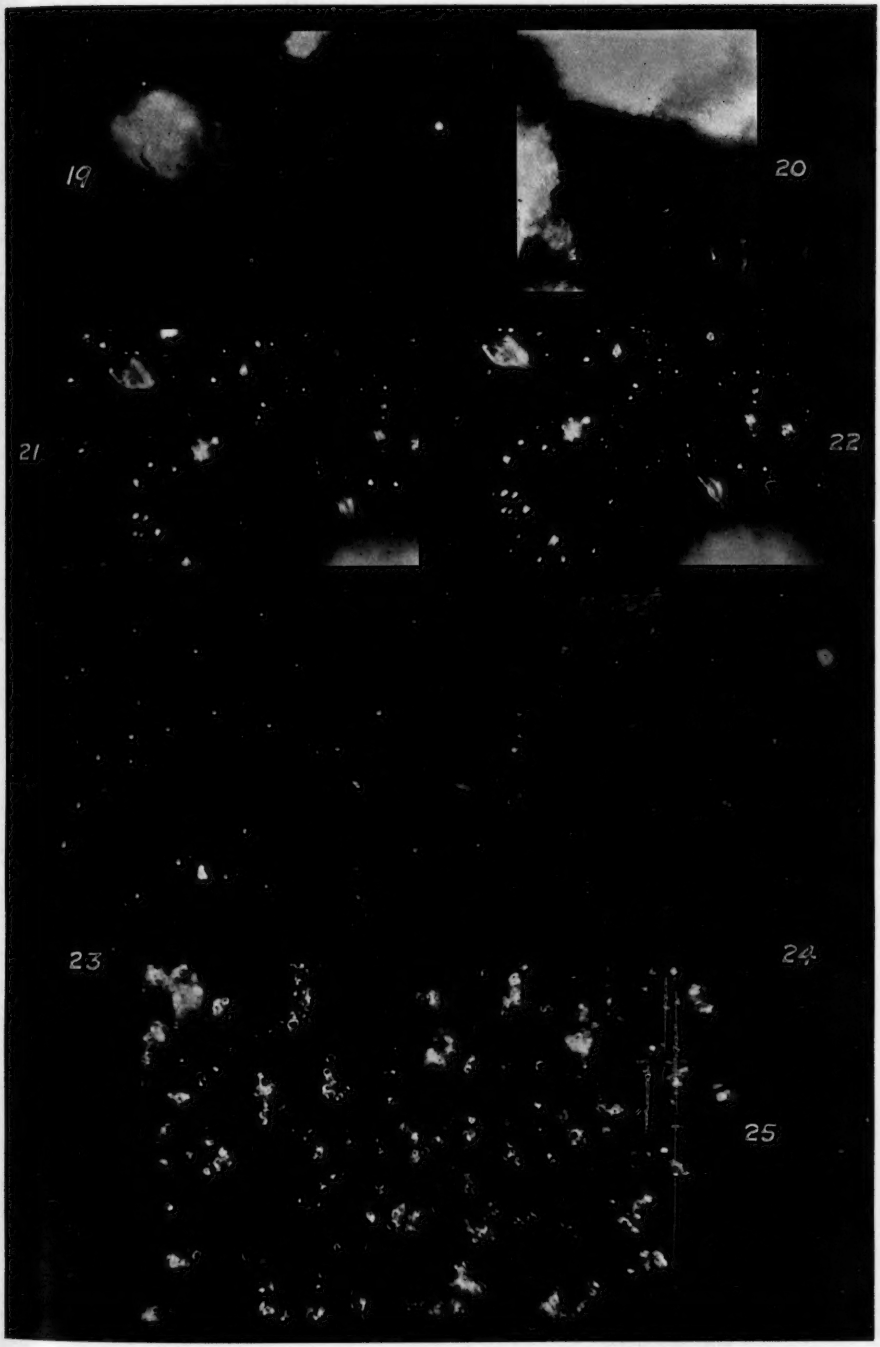
Figs. 14–18 show the multiplication of large discs from a basal sphere. Very large spheres, with or without internal organization, which were sometimes found in old cultures, are regarded as involution forms (Fig. 19).

EXPLANATION OF PHOTO-MICROGRAPHS.

FIG. 1.—Colonies on serum agar Petri dish. Incident light. Enlarged four times. × 1125.
FIG. 2.—Colonies on serum agar Petri dish in dark-ground illumination, transparent light. × 1360.
FIG. 3.—Single elementary bodies and discs. × 1140.
FIG. 4.—Single discs of different sizes floating in the liquid. × 1140.
FIG. 5.—Single discs with peripheral elementary bodies. × 1425.
FIG. 6.—Chain of elementary bodies grown from colony margin; bodies fully developed. × 1140.
FIG. 7.—Chain of elementary bodies grown from colony margin. × 1140.
FIG. 8.—Single discs growing from colony margin. × 1140.
FIG. 9.—Chain of elementary discs growing. × 1425.
FIG. 10.—Big sphere with bodies within. × 1140.
FIG. 11.—Big sphere with bodies within and elementary discs growing outside. × 1725.
FIG. 12.—The same further developed. × 1725.
FIG. 13.—The same. The big sphere is now decaying. × 950.
FIG. 14.—Big sphere, with inside organization and four grown spheres. × 1425.
FIG. 15.—The same, two days later; a fifth small sphere has grown. × 1425.
FIG. 16.—The same, one more day later; a sixth small sphere has grown. × 1425.
FIG. 17.—The same, 4 hours 15 minutes later; a seventh sphere has grown. × 1425.
FIG. 18.—The same, 6 hours later; an eighth sphere has grown. × 1425.
FIG. 19.—Giant spheres. Involution forms. × 950.
FIG. 20.—Artificial threads. × 950 reduced to × 425.
FIG. 21.—Artificial threads. Nicol prism crossed. × 950.
FIG. 22.—Artificial threads. Nicol prism parallel. × 950.
FIG. 23.—Serum bodies. × 1140.
FIG. 24.—Semi-crystalline lipoids. × 1140.
FIG. 25.—Emulsified paraffin. × 1425.



Merling-Eisenberg.



Merling-Eisenberg.

The age of the culture determines the number of stages of the life-cycle present at the same time.

If, after incubation for one or two days, the culture is kept at room temperature for a further one or two days, the agar tends to dry up, and chains of elementary bodies and discs, up to 20 in number, are of more frequent occurrence than in normal cultures. However, very long chains of twice this length have been observed on rare occasions growing on the warm stage. Good photo-micrographs of these long chains are difficult to obtain, since they float about in the fluid medium and are never in one focal plane. Sometimes the diameter of the chains of elementary bodies is so small that the individual bodies cannot be resolved, and they appear as a line or ribbon. It had been observed that these lines or ribbons subsequently developed into chains of elementary bodies. An attempt was therefore made by the application of the Azimuth diaphragm to the dark ground condenser, to resolve the ribbon into its constituent elementary bodies. The application of the Azimuth diaphragm has been described in previous papers by the present author (1930, 1932, 1935). By this method it has been possible to observe chains of elementary bodies which are so small that they appear as lines or ribbons with the ordinary dark-ground illumination. In stained preparations the presence of such lines or threads has led to much discussion.

The question of threads in cultures has been studied from another aspect. Sometimes during preparation a colony is torn so that a sector is formed similar to that shown in Fig. 20. Between the two parts, threads with a few intact elementary discs may be stretched across the gap like strings of a harp. This simile is used with the intention of illustrating a very strong suggestion of tension. The suggestion of tension in these threads led to the use of polarized light, since this is a well-known method of demonstrating a state of tension in organic or inorganic matter, as proved by Herzog (1926), Ambronn and Frey (1926), and their collaborators.

As the light coming from the dark-ground condenser is already circularly polarized, only one Nicol prism need be used as an analyser. Fig. 22 illustrates a portion of a torn colony, showing a sphere pulled apart with an intervening thread. In Fig. 21 the analysing prism is rotated 90° and the thread is nearly extinguished, leaving the parts of the sphere unaltered in their intensity. This seems to indicate that the thread is an artefact, which therefore shows the physical change due to the tension applied to it. In some cases, such a distorted sphere partly regained its round form, and this pointed to a reduction of the tension and, consequently, the degree of polarization was reduced in the same way until it became 0. It seems reasonable to infer from these physical findings that tension is the cause of these threads.

DISCUSSION.

Levaditi (1932) has described a life-cycle for rabies virus. Barnard (1925) and Dujardin-Beaumetz (1913) have shown drawings of various forms of the virus of pleuropneumonia as seen in preparations stained or otherwise, but so far as is known no previous description of the chain of events seen in living cultures over a prolonged period of time has yet been given.

In this paper, evidence has been brought forward to demonstrate a life-cycle for pleuropneumonia virus as follows :

(1) Elementary bodies ; (2) elementary discs ; (3) discs with peripheral elementary bodies ; (4) spheres with organization of elementary bodies inside ; (5) spheres breaking up and thus releasing elementary bodies. These stages are illustrated in Fig. 26.

The examination of ordinary bacteria reveals involution forms and artefacts due to the methods of preparation, and similar appearances are seen in virus cultures. The errors may arise from two sources : (1) The culture medium agar, serum and broth ; (2) the manipulation of the specimen.

It is well known that the distinction between virus bodies and serum bodies or other particles is very uncertain in stained preparations. By the dark field illumination, serum bodies are well distinguishable from discs and spheres, but not with certainty from elementary bodies. Serum bodies are shown in

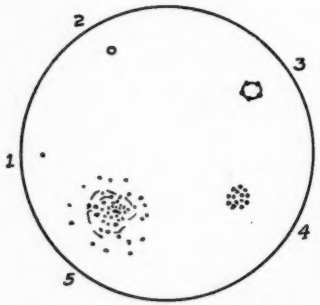


FIG. 26.—Life-cycle. × 1500.

Fig. 23. Semi-crystalline bodies of lipoidal character which might be met with in serum broth cultures can readily be recognized if their development is observed, since this takes place in a few minutes after preparing the specimen, and takes the form of branches and trees of low refractive index, as shown in Fig. 24. Other appearances which may be encountered in serum agar cultures closely resemble virus discs, but they are of such transient existence that they have been regarded as the effect of osmotic currents on the agar gel. Droplets of emulsified paraffin are of similar appearance to virus discs, as shown in Fig. 25, but their refractive index is obviously much higher, their size varies within the same field, and they do not change their appearance or position if observed over a long period of time. From what has been said, it is obvious that these errors can only be avoided by the prolonged observation of living cultures ; stained preparations at best give only disconnected pictures open to a variety of interpretations.

The nature of the much-discussed threads has been investigated by physical methods. Four different kinds of thread may be found in these specimens, originating both from the culture medium and from the organisms. (1) In serum, plasma, blood and other body fluids, very faintly diffractive threads

of various lengths may be seen, without any structure being visible, even with the Azimuth diaphragm. These threads never increase in size, and their thickness never exceeds 0·1μ. They are regarded as fibrin threads. Two forms of thread are produced by the virus: (2) chains of undeveloped and elementary bodies, (3) chains of developed elementary bodies, either growing from a colony or floating free in the fluid. Undeveloped elementary bodies forming a chain can be rendered visible by means of the Azimuth diaphragm.

(4) Threads produced by the mechanical pulling out of the virus during manipulation of the specimen. These threads are artefacts, as can be shown by the use of polarized light. They never grow, nor have they been observed in serum broth cultures.

The term of "involution form" is used in this paper to signify forms which were only observed in old cultures and did not show any further growth, such as the giant spheres shown in Fig. 17.

INSTRUMENTS.

Modifications of dark-ground illumination have been used throughout, and a list of the optical apparatus and accessories is given to facilitate repetition of the work.

Eyepieces: 10 ×, 12 ×, 14 ×, 15 ×, 20 × (Periplan, Leitz, Watson).

Objectives: 1/16 a, 1/12 a, 1/10 a (Leitz). Ultropak U.O. 75 × (Leitz). 1/12 apochromat (Watson). The objectives are fitted with calibrated irises in order to record the numerical aperture.

Condensers: Bicentric reflecting dark-ground condenser NA 1.20 (Leitz). Cassegrain NA 1.40 stopped to 1.20.

Azimuth diaphragm: Width of slit 0·5 mm. (Leitz).

Lamps: Open carbon arcs of 5, 10 and 30 amps. with aspherical aplanatic collecting lenses or collecting systems after Conradi Watson.

Carbons: Siemens A. 1.

Heat filters: Square glass jars 3 in. deep, containing a solution of Mohr salt, or distilled water. Tinted glass heat filters (Goerz, Zeiss).

Cameras: 9 × 12 cm., 4·5 × 6 cm. and Leica size (Leitz).

Hot stage: For microscope (Leitz).

Photographic plates: Rapid Chromatic and Double X Press (Ilford). The optical systems were carefully centred, since this is essential for success.

SUMMARY.

A well-defined life-cycle for the pleuropneumonia virus in culture is described and illustrated.

I wish to thank Prof. J. McIntosh for direction and advice in this research. The earlier part of the investigation was carried out with the aid of a grant from the Academic Assistance Council, and I am indebted to Prof. Samson Wright for providing facilities for this portion of the work.

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ADSORPTION AND ELUTION OF THE ROUS SARCOMA AGENT

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THE adsorption of the active agent of the Rous chicken sarcoma No. 1 was first reported by Lewis and Andervont (1926) and Milone (1928). Fraenkel, Mislowitzer and Simcke (1928) were successful in obtaining tumours by adsorption on caseinogen, kieselguhr and kaolin, and Leitch (1928) obtained active eluates from a kaolin adsorbate. Fraenkel and Mislowitzer (1929), using membrane filtrates of Rous sarcoma extracts, obtained active eluates by elution of adsorbates on alumina with phosphate buffers. The even distribution of activity between adsorbate and supernatant showed that this method of purification was not highly specific under the conditions used, but Fraenkel and Mislowitzer (1930) reported an equal proportion of tumours with filtrate and eluate. After trypsinization a higher percentage of tumours was obtained in both filtrate and eluate. Maschmann and Albrecht (1931) found that the Rous agent was adsorbed very well on alumina C and on ferric hydroxide at pH 6·0, and they obtained active eluates with alkaline ammonium phosphate and with 0·05 p.c. ammonia solution.

Murphy, Claude, Sturm and Helmer (1932) state that adsorption of Rous extract with alumina leads to removal of an inhibitor by the solid phase, leaving a supernatant more active than the original filtrate. They consider that though some of the agent is adsorbed, the greater proportion remains in the supernatant. This point of view is developed by Claude and Murphy (1933) who add that alumina adsorption of an extract made from an inactive sample of dried Rous sarcoma will give an active supernatant. Elution of

such adsorbates with alkaline buffers gave eluates containing an inhibitor, and they believe that the specific action of alumina is the adsorption of inhibitory substance rather than tumour agent. In a recent paper by Claude (1935), however, an equal distribution of activity between supernatant and adsorbate was reported, and the eluate had half the potency of the original extract, in spite of the fact that as much as 1·3 p.c. of alumina was used for adsorption. Pirie (1931) found that if the concentration of alumina were greater than 0·6 p.c. the agent could not be eluted, and stated that the best concentration of alumina for the preparation of active eluates was 0·15 p.c.

Several workers have suggested that the Rous agent is of an acid nature, or is bound to material having acid properties. Sittenfield, Johnson and Jobling (1931) separated the agent from the inhibitor by repeated precipitations at pH 4·0 and elution of the precipitates at pH 8·0. The inhibitor was obtained in the supernatant from the first precipitation at pH 4·0 and the agent in the precipitate, which was further purified by repeated precipitation at pH 4·0 and elution at pH 8·0. A great many other cases have been reported where the active material has been precipitated by acidification below pH 4·7, but the difficulty has always been that large amounts of inert protein are precipitated as well.

Adsorption and elution with buffers of varying pH was a promising variant of this mode of attack, and Pirie (1931) found that adsorption on alumina C was much more efficient at pH 7 or 9 than at pH 5. Elutions were performed in each case at pH 9·2 with phosphate buffer. Nakahara and Nakajima (1933), using alumina B, reported that adsorption was poor at pH 4 to 6, but good at pH 7 to 10. Elution was successful at pH 8·7 with alkaline sodium phosphate or $M/25$ ammonia, but active eluates could not be obtained between pH 4 and 6. These authors reported complete adsorption and complete elution in the most favourable circumstances, but we have been unable to confirm this observation.

Although active eluates usually contain detectable protein, they do not possess the viscous characteristics of the Rous extract which remain in the supernatant after adsorption, and Pirie (1931) and ourselves have obtained tumours with eluates which gave no chemical indication of protein. Eluates made at pH 4·7 or 6·0 from filtered extracts adsorbed at pH 4·7 or 6·0 seldom contain more than small traces of protein, but such eluates give a low proportion of tumours. However, we believed that we might best approach the problem of separation of agent and inhibitor and purification of the agent by means of adsorption at varying pH and elution of each adsorbate by a series of different buffers, and at the same time we investigated the efficiency of adsorption and elution with the Wilstätter aluminas A, B, C and D.

In the latter experiments adsorption was carried out on extracts made with $M/15$ phosphate buffer at pH 4·7 to 6·1, and eluates were made with similar buffer at pH 8·3 to 8·7. Tumours were obtained in a high proportion of cases with each of the adsorption materials used, but on the whole the C and D reagents gave the best results. In addition to being somewhat less efficient, the A and B reagents were much less easy to work with, owing to the "gummy" nature of the adsorbate, which made washing difficult. The C reagent was much the best from this point of view because, after one washing

with water, the alumina broke up into a flocculent form which was very easily manipulated. There appeared to be no appreciable difference in the activity of the supernatants from the various adsorption materials. Alumina C was used in all pH experiments.

The hope that a process of differential adsorption and elution would lead to a clean separation of agent and inhibitor has not been fulfilled, but it is possible to say that alkaline eluates made from any adsorbate are more efficient than acid eluates, and that adsorption is most efficient at pH 6·0. In general we have found that the most potent eluate is one made from material adsorbed at pH 6·0 and eluted at pH 8·4. Part of the agent is always left in the supernatant and part of the adsorbed agent remains in the residue after elution, so it can hardly be hoped that any considerable concentration of the agent can be obtained by these means.

Extracts made at pH 7·5 and 8·4 are more potent than those made at pH 4·7 and 6·0, and the supernatants from adsorption at pH 7·5 and 8·4 gave tumours earlier than those from adsorbates made at pH 4·7 and 6·0. The differences in these cases were not, however, very clear-cut or consistent. These observations, together with the relatively high potency of alkaline eluates, contrast with the statement of Claude and Murphy (1933) that the inhibitor is removed from the solution by adsorption on alumina C and can be eluted at alkaline pH. This difference in results may be due to the difference in amount of adsorbent used or to differences in the properties of the tumour. We have sometimes found it impossible to produce tumours from eluates and at other times, particularly in April, May and June, the eluates have been very potent, but at no time have we observed any evidence of the presence of more inhibitor in the alkaline eluates than in the acid ones.

EXPERIMENTAL.

The extracts were made by grinding chopped tumour tissue with sand and extracting for 30 minutes in the refrigerator with ten times the tissue weight of *M*/15 phosphate buffer. The extracts were centrifuged at 3000 r.p.m. for 30 minutes ; the supernatant was poured off and re-centrifuged for 15 minutes. The resulting liquid was cloudy, but further centrifugation gave only a small deposit of cell débris in which no whole tumour-cells could be detected microscopically. The centrifugates were sometimes filtered through 0·7μ Bechold membranes, but, apart from causing some decrease in potency, this operation did not alter the results. In one experiment the material was kept saturated with toluene at all stages, but there was no significant difference in results as compared with those of a parallel experiment without toluene. The alumina adsorption reagents were made according to Kraut (1925) and contained about 3·0 mg. alumina per ml. Adsorption was carried out with equal parts of extract and alumina suspension and continued for 15 minutes. The adsorbate was washed three times with distilled water. Elutions were carried out with an amount of phosphate buffer equal to the volume of extract from which the adsorbate had been made, and separated on the centrifuge after 15 minutes. At every stage the material was kept as far as possible at 0° C.

The assessment of results has presented some difficulty. We examined

the chickens every other day and noted the time after injection at which the tumour first became palpable, but this is not in itself a reliable index of potency, since the rapidity of appearance of a tumour varies with the exact site of injection and the individual susceptibility of the bird. The weight of the tumour at autopsy suffers from similar objections, and it is often difficult to decide whether a given piece of tissue should be regarded as tumour or not. We have used as an index of potency an expression involving both the weight of the tumour at autopsy and the number of days before the tumour became palpable. In each bird, injected in both breasts and both legs, the weights of the respective tumours in grammes have been divided by the number of days elapsing between injection and detection of the tumour. Each figure so obtained has been expressed as a percentage of the largest figure in that bird An example is given in Table I.

TABLE I.—*Method of Calculation of Index of Potency.*

	Left. leg.	Right leg.	Left breast.	Right breast.
Days before tumour palpable	10	12	20	25
Weight of tumour at autopsy (g.)	50	36	40	25
Weight/days	5	3	2	1
Index	100	60	40	10

It is realized that this index gives at best an approximate idea of the relative potency of the material injected into any given bird, and that it tends to exaggerate some of the faults of both of its component criteria, but on the one hand it takes into account the high potency of an injection which gives rise to a tumour very rapidly, and on the other makes allowance for a tumour which, because of its mode of growth, may be quite large and yet not be detected until autopsy.

In the experiments with the four Wilstätter adsorbents A, B, C and D, 5 ml. of extract was adsorbed with 5 ml. of alumina suspension for 15 minutes, the centrifuged adsorbate washed three times with 5 ml. distilled water, and eluted for 15 minutes with 5 ml. phosphate buffer. Four ml. of the eluate was

TABLE II.—*Adsorption and Elution of Rous Sarcoma Agent with Alumina.*

Date. Adsorption material.	Potency index. A.		B.		C.		D.	
Material injected.	SN.	EL.	SN.	EL.	SN.	EL.	SN.	EL.
2.viii.34	..	100	..	16	..	45	..	14
22.viii.34 (1)	..	0	..	0	..	35	..	100
22.viii.34 (2)	..	2	..	3	..	17	..	100
14.xii.34	0	13	0	19	0	100	0	63
8.iii.35	15	0	100	0	62	0	45	0
16.iv.35 (1)	52	30	10	28	100	100	4	53
16.iv.35 (2)	..	100	..	55	..	25	..	100
Mean	..	35	..	17	..	46	..	61

SN. = Supernatant; EL. = Eluate.

TABLE III.—*Adsorption and Elution of Rous Sarcoma Agent with Alumina.*
Date: 16.iv.35 (1).

Adsorption material	A.			B.			C.			D.		
Material injected	SN.	EL.	RES.	SN.	EL.	RES.	SN.	EL.	RES.	SN.	EL.	RES.
Palpable days after injection	13	17	13	15	17	13	13	15	15	17	15	15
Weight of tumour at autopsy (g.)	9·5	34	24	2	31	27	19	98	17·5	1	53	11

SN. = Supernatant; EL. = Eluate; RES. = Residue after elution.

injected, being equivalent to 400 mg. of tumour tissue. The results are given in Table II, and the detailed protocol of one experiment in Table III.

For the adsorption and elution experiments at various values of pH, a 1/10 extract was made with *M*/15 phosphate buffer. Twenty-five ml. of extract was adsorbed with 25 ml. of alumina C for 15 minutes, and after centrifuging it was usually found that the pH had not altered a great deal. In a typical experiment where the pH of the buffers used for extraction was originally 4·7, 6·0, 7·5 and 8·3, the values for the supernatants after adsorption were 5·8, 6·2, 7·5 and 8·2. In the tables the original pH of the buffer has been given. The centrifuged adsorbate was washed twice with 25 ml. distilled water and mixed thoroughly with 25 ml. water, divided equally into four or five parts and each portion centrifuged. These portions were then eluted with their appropriate buffer. In each case an amount of eluate was injected equivalent to 400 mg. of the original tumour tissue. As an experiment of this kind lasted about seven hours, care was taken to keep all materials in ice wherever possible. In some of the experiments reported the eluates made at the two lower pH's have been injected in the legs and the other two in the breast. In the others the more alkaline eluates were injected in the breasts and the more acid ones in the legs. A summary of results is given in Table IV and a detailed protocol of one experiment in Table V.

SUMMARY.

An attempt has been made to purify the Rous sarcoma agent, and particularly to separate agent and inhibitor, by the selection of the most suitable form of alumina for adsorption and the use of buffers of varying pH for adsorption and elution. The best adsorption materials were found to be the Wilstätter C and D aluminas and the most potent eluate was one made from material adsorbed at pH 6·0 and eluted at pH 8·4. In general, the most potent eluates were those made at pH 7·5 and 8·4. Since a considerable amount of the agent is left in the supernatant during adsorption, and more is left in the residue during elution, no concentration of the agent was obtained by these means.

We wish to acknowledge the collaboration of Mr. J. W. Walker in this work, which has been done with the aid of the Cancer Research Fund of the Westminster Hospital. We are much indebted to Dr. C. F. Andrewes of

TABLE IV.—*Adsorption and Elution of Rous Sarcoma Agent with Alumina C and Phosphate Buffers of Varying pH.*

Extracted and adsorbed at pH.	Potency index. 4·7.				6·0.				7·5.				8·4.			
Eluted at pH	4·7.	6·0.	7·5.	8·4.	4·7.	6·0.	7·5.	8·4.	4·7.	6·0.	7·5.	8·4.	4·7.	6·0.	7·5.	8·4.
Date.																
17.v.34	1·4	1·7	100	76	0	0	100	67	0	33	33	100	7·0	33	100	100
25.v.34	0	34	100	12	0	19	73	100	0	0	39	100	0	0	100	39
7.vi.34	0	0	100	91	0	16	100	93	0	Tr.	100	76	6·0	6·4	100	98
2.viii.34	0	0	..	100	..	..	..	..	..	..	..	..	..	..	..	..
14.ii.35	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	100
28.iii.35	0	0	0	0	0	0	20	100	0	0	0	0	0	0	0	0
25.iv.35	0	0	26	100	Tr.	4·8	56	100	0	0	90	100	0	0	50	100
Mean	0·2	5·1	54	54	0	6·7	58	93	0	6·6	44	63	2·2	6·6	58	73

Tr. = Trace of tumour.

TABLE V.—*Adsorption and Elution of Rous Sarcoma Agent with Alumina C and Phosphate Buffers of Varying pH.*

Date: 7.vi.34.

pH extracted and adsorbed	Filtrate. 4·7.	6·0.	7·5.	8·4.	Supernatant. 4·7.	6·0.	7·5.	8·4.	Adsorbate. 4·7.	6·0.	7·5.	8·4.
Palpable days after injection	11	11	7	7	14	14	11	11	11	11	11	11
Weight at autopsy (g.)	..	..	..	..	2·2	2·2	7·2	23·8	9·5	3·0	8·5	11·5

Eluates.

pH extracted and adsorbed	4·7.				6·0.				7·5.				8·4.			
Eluted at pH	4·7.	6·0.	7·5.	8·4.	4·7.	6·0.	7·5.	8·4.	4·7.	6·0.	7·5.	8·4.	4·7.	6·0.	7·5.	8·4.
Palpable days after injection	∞	∞	14	14	∞	16	14	14	∞	21	14	14	16	14	16	14
Weight at autopsy (g.)	0	0	88	80	0	5	28	26	0	Tr.	30	23	2	2	35	30

Tr. = Trace of tumour.

the National Institute for Medical Research, Hampstead, for supplying us with the strain of Rous sarcoma.

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FURTHER OBSERVATIONS ON THE PROPERTIES OF THE VI ANTIGEN OF *B. TYPHOSUS* AND ITS CORRESPONDING ANTIBODY.

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SOME of the properties of the Vi antigen of *B. typhosus* and of its corresponding antibody have been described in previous papers (Felix and Pitt, 1934*a* and *b*; Felix, Bhatnagar and Pitt, 1934). In the present communication the *rôle* played by the Vi antibody in the phagocytosis of *B. typhosus* is demonstrated, and an account is given of an inquiry into some puzzling differences in its behaviour in immune sera prepared with live or with killed organisms respectively.

VI ANTIBODY AND PHAGOCYTOSIS OF *B. TYPHOSUS*.

It has been shown in previous papers that the presence of Vi antigen in virulent strains of *B. typhosus* and its absence from strains of low virulence have a far-reaching effect on the course of phagocytosis. Inagglutinable strains, which are rich in Vi antigen, are highly resistant to phagocytosis; agglutinable strains, which are devoid of Vi antigen, are highly susceptible (Bhatnagar, 1935). Highly virulent and inagglutinable strains, when grown at temperatures unsuitable for the development of Vi antigen, are deprived

at the same time of their virulence, their inagglutinability by O antibody and their resistance to phagocytosis (Felix, Bhatnagar and Pitt, 1934).

In view of the powerful invasion-suppressing action of the Vi antibody established in mouse experiments, it seemed desirable to study the effect of this antibody on phagocytosis. The immune sera used in this investigation were rabbit sera which had been employed in previous experiments on Vi agglutination and on passive immunization of mice. The technique of the phagocytosis tests was as described by Bhatnagar (1935).

TABLE I.—*The Rôles of O and Vi Antibodies in Phagocytosis.*

Immune sera in dilution 1 in 500 plus complement (normal serum No. 20 in dilution 1 in 10).		Titre of antibodies estimated by agglutination.		Phagocytosis of strains.		
Rabbit No.	Immunized with live strains.	O.	Vi.	Giglioli.	Rawlings.	H901.
71	Ty 8	5000	0	38/34	438/78	610/92
72	Ty 901	10,000	0	56/42	688/96	860/100
73	Rawlings	20,000	0	80/64	750/100	890/100
62 (absorbed serum)	Ty 2	0	400	920/100	242/50	402/82
		Controls.				
Complement alone (normal serum No. 20 in dilution 1 in 10)		20	0	10/10	256/72	420/84
Saline				0	4/4	1

Note.—The numerators of the fractions denote the number of organisms phagocytosed by 100 leucocytes, the denominators, the percentage number of cells taking part.

In the experiment summarized in Table I, four immune sera, all from rabbits which had been immunized with suspensions of live *B. typhosus*, were tested for their sensitizing power against the highly virulent strain "Giglioli" and against two strains of low virulence, "H901" and "Rawlings". Three of the immune sera were rich in O antibody but contained no Vi antibody, since the strains used for the immunization of these rabbits were of the agglutinable type, which is devoid of the Vi antigen. The serum No. 62 was a pure Vi serum, from which the H and O antibodies had been removed by absorption with the highly agglutinable strain "H901". In this and the following tables the H titres are not recorded, since it is known that the H antibody has no appreciable effect on phagocytosis (Braun and Nodake, 1924; Bhatnagar, 1935).

Table I shows that the three high-titre O antisera had a marked sensitizing effect on all the three strains tested. There is, of course, a very great difference in the degree of phagocytosis attained with the resistant, virulent strain "Giglioli" as compared with the sensitive, avirulent strains "H901" and "Rawlings". But with both types of strain the figures showing phagocytosis

in the presence of complement and O immune serum were invariably higher than those recorded for the complement control (normal serum alone), indicating the degree of sensitization due to the action of the O immune serum.

Entirely different effects were observed with the pure Vi serum No. 62. It exerted a powerful sensitizing action on the virulent strain "Giglioli", which contains Vi antigen, but had no effect at all on the avirulent strains "H901" and "Rawlings", which do not possess this antigen. The phagocytosis-promoting effect on strain "Giglioli" of the pure Vi serum No. 62 is therefore clearly another manifestation of the specific reaction between Vi antigen and Vi antibody, falling into line with those previously demonstrated by agglutination, precipitation, and active and passive immunization of mice.

The potency of the Vi antibody in its sensitizing activity appears particularly striking in view of its comparatively low titre in the agglutination reaction, which, in the absence of some more adequate method, is used for the quantitative estimation of the Vi antibody. In contrast with the poor sensitization of the virulent strain "Giglioli" attainable by O antisera of very high agglutinating titres, the action of the Vi antiserum can justly be stated to be enormously powerful. This is in complete agreement with what is known with regard to the relative potency of O and Vi antibodies in protection against infection with virulent strains of *B. typhosus*.

TABLE II.—*The Summation of the Sensitizing Effects of O and Vi Antibodies.*

Immune sera in dilution 1 in 500 plus complement (normal serum No. 24 in dilution 1 in 10).		Titre of antibodies estimated by agglutination.		Phagocytosis of strain.
		O.	Vi.	Giglioli.
No. 62	Absorbed	0	400	850/100
	Unabsorbed	5000	400	960/100
No. 74	Absorbed	0	200	265/96
	Unabsorbed	2000	200	310/98
No. 67	Absorbed	0	100	160/74
	Unabsorbed	5000	100	240/86
Controls.				
Complement alone (normal serum No. 24 in dilution 1 in 10)		20	0	12/12
Saline				0

Note.—Titre O = a negative result in agglutination in dilution 1 in 10.

Table II shows that there is a summation of the sensitizing effects of O and Vi antibodies when the two are allowed to act simultaneously on a strain of the virulent type, possessing the corresponding two antigens. Three immune sera, all containing O and Vi antibodies, were tested for their sensitizing power, (*a*) unabsorbed, and (*b*) after their O antibody content had been

removed by absorption. The figures for phagocytosis were in each case lower with the absorbed than with the unabsorbed samples of serum. The difference, though comparatively small, was in each instance quite significant, and its numerical value was in the range of those figures which in Table I indicated the sensitizing effects of the immune sera on the virulent strain Giglioli.

Table II also shows that the power of promoting phagocytosis and the Vi titres of the sera tested run parallel. Analogous results were obtained in numerous experiments with these and other rabbit immune sera which had different titres of Vi agglutination, and were used in the phagocytic mixtures in final dilutions ranging from 1 in 10 to 1 in 2000. In this respect, too, there was complete agreement between the potency of the Vi antisera in promoting phagocytosis, and in protective action against infection of mice with virulent strains of *B. typhosus*.

Certain experiments were especially designed to decide whether the mechanisms involved in sensitization by Vi and O antibodies respectively were similar or different in nature. It was thought possible that the part played by the Vi antibody in the phagocytosis of virulent strains was to neutralize the Vi antigen present in these strains and thereby to annul their resistance to the sensitizing action of the O antibody, which also was invariably present in the phagocytic mixtures, either as immune O antibody in the Vi + O immune serum, or as natural O antibody in the fresh normal serum that served as complement. No experimental evidence could, however, be produced in support of this hypothesis.

TABLE III.—*The Rôle of Complement in Sensitization by O and Vi Antibodies.*

Immune sera in dilution 1 in 500.	Titre of antibodies.		Complement (normal serum No. 20 in dilution 1 in 10).	Phagocytosis of strains.		
	O.	Vi.		Ty 2.	Rawlings.	H901.
No. 62	0	400	Fresh	880/100	254/76	410/94
(absorbed)			Inactivated	6/6	8/8	5/5
No. 67	0	100	Fresh	145/68	250/78	395/82
(absorbed)			Inactivated	4/4	5/5	8/8
No. 73	20,000	0	Fresh	70/64	720/98	870/100
			Inactivated	2/2	68/42	78/48
No. 64	20,000	0	Fresh	64/56	690/94	860/100
			Inactivated	1	52/38	64/48
			Controls.			
Normal serum (complement) No. 20 in dilution 1 in 10	20	0	Fresh	8/8	240/68	390/88
			Inactivated	0	4/4	3/3
Saline				0	3/3	1

Table III shows that for the phagocytosis-promoting action of the Vi antibody the presence of complement is indispensable. It is seen that by inactivating the complement the sensitizing activity of the Vi antibody is suppressed even more completely than is that of the O antibody. Whereas the two O antisera (Nos. 73 and 64), unaided by complement, were still capable of sensitizing the strains "H901" and "Rawlings" quite significantly, the two pure Vi antisera Nos. 62 and 67 were, under similar conditions, almost entirely devoid of the capacity of sensitizing the virulent strain Ty 2.

THE SENSITIZING AND PROTECTIVE POWER OF THE Vi ANTIBODY IN THE SERUM OF RABBITS IMMUNIZED WITH FORMOLIZED EXTRACTS.

In the experiments described in the preceding section only immune sera were used which had been prepared by inoculating into rabbits suspensions of *B. typhosus* in the living state. Later Vi antisera were available from rabbits immunized by intravenous inoculation of saline extracts from virulent strains of *B. typhosus* which had been sterilized by the addition of 0·2 p.c. formalin. High-titre Vi antisera were obtained by this method, the titres of Vi agglutination being equal to or even higher than those previously observed in rabbits immunized with living virulent bacilli (Felix, Bhatnagar and Pitt, 1934).

When these sera were tested for their sensitizing power in phagocytic experiments, it soon became clear that in this respect the Vi antibody resulting from immunization with formolized extracts was very much inferior to the Vi antibody elaborated in response to immunization with living bacilli. Table IV gives a sample experiment. The details of the preparation of formolized extracts have been described previously (Felix, Bhatnagar and Pitt, 1934). It may be added here that the rabbits Nos. 79 and 82 were immunized with a mixture of equal parts of extracts from the three virulent strains "Ty 2",

TABLE IV.—*Sensitization by Vi Antibody Produced by Immunizing Rabbits with Formolized Extracts.*

Immune sera in dilution 1 in 500 plus complement (normal serum No. 20 in dilution 1 in 10).		Titre of antibodies estimated by agglutination.		Phagocytosis of strains.	
Rabbits immunized with—	No. of serum.	O.	Vi.	Ty 2.	Watson.
Formolized extracts	79 V	10,000	600	160/76	210/82
	82 V	5000	600	126/68	172/76
Strain Ty 901 live	72	10,000	0	88/72	130/78
Strain Ty 2 live	62	5000	400	860/100	930/100
		Controls.			
Complement (normal serum No. 20 in dilution 1 in 10)		20	0	8/8	12/12
Saline				0	0

"Giglioli" and "Watson". The animals received four intravenous inoculations at intervals of two to four days. The first dose was given two days after the formolized extracts had been prepared, the other injections followed on the 4th, 6th and 10th day, respectively.

It is seen from Table IV that the two sera from extract immunization (Nos. 79V and 82V) contained Vi antibody, demonstrable by agglutination, to a titre higher than that of the serum No. 62, which was from a rabbit immunized with living bacilli. Yet, the numbers of organisms of the two virulent strains "Ty 2" and "Watson", that were phagocytosed in the presence of the sera Nos. 79V and 82V, were very much lower than those recorded for the serum No. 62. The phagocytic figures for the control serum No. 72, which was an O antiserum devoid of Vi antibodies, indicate the degree of phagocytosis due to sensitization by the O antibodies that also were present in the three Vi + O immune sera under test. If these control figures are taken into account the discrepancy between the high sensitizing power of the serum No. 62, and the poor effects of the sera Nos. 79V and 82V is made still more conspicuous.

A similar discrepancy was also found between the activity of the Vi antisera from extract immunization and of those from immunization with living bacilli, when the two types of serum were compared for their potency in protective action against infection with a highly virulent strain of *B. typhosus*. An illustrative experiment is summarized in Table V.

TABLE V.—*Protective Values of Vi Antisera Prepared by Immunization with Living Bacilli and with Formolized Extracts.*

Immune sera. Rabbits immunized with—	No.	Titre of antibodies estimated by agglutination. O.	Vi.	Dose of serum in c.c.	Number of mice. Tested.	Survived.
Formolized extracts	79 V	10,000	600	1·0	4	1
	82 V	5000	600	1·0	6	2
Living bacilli (strain Ty 2)	61	5000	200	1·0	4	4
	62	5000	400	0·5	4	4
				0·25	4	3
Test dose		3 M.L.D. (240×10^6 organisms of strain Ty 2).				

The three Vi antisera which had been employed in the preceding phagocytic experiment and another rabbit serum (No. 61), obtained after immunization with living bacilli, were tested for their protective values in passive immunization of mice. The technique of the protection tests was that described in previous papers (Felix and Pitt, 1934*a* and *b*). The Vi antisera produced by immunization with formolized extracts had very much less protective power than the sera produced by immunization with living organisms.

The surprising fact was thus established that the Vi antibody resulting from immunization with formolized extracts of virulent cultures of *B. typhosus*

is not in all respects identical with the Vi antibody formed in response to immunization with the live organisms. Both the protective power and the phagocytosis-promoting activity of the former are very much inferior to those of the latter, though in Vi agglutination no difference has so far been discernible between the two types of serum. In fact the titres of Vi agglutination may even be higher in the sera from extract immunization.

We are not aware of any published data that could be interpreted as providing an analogy to the facts established in these experiments. It would appear that the most plausible explanation of the phenomenon would be the assumption that the Vi antigen of *B. typhosus*, by virtue of its extreme lability, is capable of undergoing, as a result of the treatment with 0·2 p.c. formalin, some slight chemical alteration, which finds its expression in the formation of a slightly different antibody. This assumption would receive support if it were shown that the relative ineffectiveness of the altered Vi antibody in mouse-protection and phagocytic experiments is paralleled by a similar ineffectiveness in complement-fixation and bactericidal tests. This would indicate that the altered Vi antibody is deficient in the capacity for inducing all those immunity reactions that depend on the active participation of complement. It is proposed to test this point in future experiments.

THE SUSCEPTIBILITY OF THE Vi ANTIGEN TO THE ACTION OF PHENOL AND FORMALIN.

From certain data published in the previous papers of this series it is already known that the Vi antigen of *B. typhosus* is in some respects even more labile than the H antigen, which in its turn has been styled the "labile" antigen as contrasted with the "stable" O antigen. To quote two examples only: the H antigen requires for the destruction of its agglutinogenic activity exposure to higher temperatures than does the Vi antigen; temperatures during growth which permit of abundant development of H antigen markedly inhibit the formation of Vi antigen in the cultures. Following the puzzling observations recorded in the preceding section, the effects on the Vi antigen of exposure to the action of formalin and phenol were more closely investigated. The two disinfectants were tested each in one concentration only, viz. 0·2 p.c. formalin and 0·5 p.c. phenol—the concentrations most commonly employed in bacteriological routine.

The point first tested was whether the agglutinability by Vi antiserum of suspensions of bacilli containing Vi antigen was impaired as a result of the treatment with formalin or phenol. Kauffmann (1935) has already established that formolized suspensions could be used instead of living bacilli for the demonstration of Vi agglutination.

In our experiments the three virulent strains "Ty 2", "Watson" and "Giglioli" were employed. The cultures of the first two strains were highly motile and rich in H antigen, whereas the culture of the strain "Giglioli" was almost entirely devoid of flagella and H antigen. Agar cultures grown in Roux bottles for 24 hours at 37° C. were suspended in saline, standardized by opacity to contain 16,000 million organisms per c.c., and divided into three

equal portions. Equal quantities of (*a*) saline, (*b*) saline + 0·4 p.c. formalin, (*c*) saline + 1 p.c. phenol were added to the three portions respectively, the first of which served as a control. One drop of these suspensions, which now contained 8000 million organisms per c.c., when added to 1 c.c. of the serum dilutions produced the standard opacity required for the actual agglutination test. The three suspensions were kept in the dark at room temperature for two days, when the formolized and phenolized suspensions proved to be sterile. They were then stored in the cold room and tested for their agglutinability by pure H, O and Vi antisera, first at shorter and then at longer intervals extending over a period of five weeks. The results of these agglutination tests may be summarized as follows :

1. The agglutinability by Vi antiserum of all the suspensions of the three strains was well maintained. With the formolized suspensions the readings throughout remained almost identical with those recorded for the saline controls ; with the phenolized samples there was a slight, almost negligible, diminution in the sensitiveness of the suspensions, noticeable immediately after they had been prepared.

2. The inagglutinability by O antiserum remained unaltered by formalin, but was partially annulled by phenol. Again, the effect produced by the phenol was observable immediately after the preparation of the suspensions.

3. Both the formolized and the saline suspensions of the non-motile strain " Giglioli " also lost part of their inagglutinability by the O antibody, apparently because the inhibiting effect of formolized H antigen does not appear with the non-motile strain (Felix and Olitzki, 1928).

The three suspensions were re-tested after storage for three months. The agglutinability by Vi antiserum of the formolized suspensions was found to be still unimpaired, whereas that of the phenolized and of the control suspensions were reduced to about half of the original titres.

Next the agglutinogenic value of the Vi antigen in phenolized and formolized suspensions was investigated. Suspensions of the three virulent strains of *B. typhosus*, containing 2000 million organisms per c.c., were sterilized by the addition of 0·5 p.c. phenol and 0·2 p.c. formalin respectively. Sterilization was accomplished within two days at room temperature. The suspensions were then stored in the cold room and used for the immunization of rabbits by the intravenous route, according to the following scheme :

1st dose,	2000 million organisms, 3 days after preparation of the vaccines.
2nd dose,	3000 million organisms, 7 days after preparation of the vaccines.
1st bleeding	. . . . 7 days after second dose.
3rd dose,	3000 million organisms, 16 days after preparation of the vaccines.
2nd bleeding	. . . . 7 days after third dose.

The titres of H, O and Vi agglutination observed with the sera from the two series of bleedings varied but slightly. In Table VI are reproduced those recorded with the samples from the later bleedings.

It is seen from Table VI that none of the rabbits immunized with the phenolized suspensions produced any trace of Vi antibody, whereas all the rabbits immunized with formolized suspensions elaborated high-titre Vi antisera. No difference was observed between the two series with regard to the titres of H and O agglutinins. This does not contradict the observation recently published by Stuart and Krikorian (1934), who showed that phenol has some deleterious effect on the O antigen of *B. typhosus*. These authors found in experiments on rabbits and man that antityphoid vaccines killed by heating at 60° C. and preserved by the addition of 0·5 p.c. phenol were less efficient in inducing the formation of O agglutinins than were vaccines to which no phenol had been added as preservative. Our experiments were not designed to reproduce the effect observed by Stuart and Krikorian. We used considerably greater doses of vaccines and administered them by the intravenous instead of by the subcutaneous route, and this obviously accounts

TABLE VI.—*Vi, O and H Agglutination with Sera from Rabbits Inoculated with Phenolized and Formolized Suspensions.*

Type of antibody.	Titre of agglutination. Rabbits immunized with suspensions of strains.					
	Killed by 0·2 p.c. formalin.			Killed by 0·5 p.c. phenol.		
	Ty 2.	Giglioli.	Watson.	Ty 2.	Giglioli.	Watson.
Vi	400	200	1000	0	0	0
O	20,000	20,000	10,000	20,000	20,000	20,000
H	20,000	5000	20,000	10,000	20,000	5000
No. of rabbit	111	112	113	108	109	110

Note.—Titre O = a negative result in dilution 1 in 10.

for the abundant production of O agglutinins in all the rabbits tested. For the purposes of the present investigation the interest of the experiment illustrated in Table VI lies in the demonstration of the extreme susceptibility of the Vi antigen to the action of phenol as contrasted with its relative insusceptibility to formalin.

Finally, the protective values of the rabbit sera used in the preceding experiment were compared with those of immune sera produced by immunization with living bacilli. The results of one of these mouse experiments are recorded in Table VII.

The sera from the three rabbits inoculated with phenolized vaccines had no protective action, since they were devoid of Vi antibody. The two immune sera Nos. 111 and 62 again showed that striking discrepancy between titre of Vi agglutination and potency in protective action, which has been discussed in the preceding section. Thus the Vi antibody elaborated in response to the inoculation of a formolized vaccine was found to be deficient in protective power much in the same way as the Vi antibody produced by immunization with formolized extracts.

TABLE VII.—*Protective Values of Immune Sera from Rabbits Inoculated with Phenolized, Formolized and Live Suspensions of* B. typhosus.

Immune sera.			Titre of antibodies estimated by agglutination.		Dose of serum in c.c.	Number of mice.	
Rabbit No.	Immunized with strain.	Type of vaccine.	O.	Vi.		Tested.	Survived.
108	Ty 2	Phenolized	20,000	0	1·0	4	0
109	Giglioli	Phenolized	20,000	0	1·0	4	0
110	Watson	Phenolized	20,000	0	1·0	4	0
111	Ty 2	Formolized	20,000	400	0·8	4	2
					0·4	4	1
					0·2	4	0
62	Ty 2	Live	5000	400	0·8	4	4
					0·4	4	4
					0·2	4	4

Test dose . . . 3 M.L.D. (240 × 10^6 organisms of strain Ty 2).

DISCUSSION.

It has been shown in previous experiments (Felix and Pitt, 1934*b*) that in active and passive immunization against highly virulent strains of *B. typhosus* the Vi antigen and Vi antibody are of very much higher protective value than are the smooth O antigen and its serum counterpart. This fact came as something of a surprise in view of the great immunological significance now generally assigned to the O antigen and O antibody (Felix, 1924; Felix and Olitzki, 1926; Arkwright, 1927; Topley, 1929; Schütze, 1930). The results of the present investigation corroborate the earlier observation, and also throw some light on the intricate mechanisms involved. The outstanding power of the Vi antibody of promoting phagocytosis of highly virulent strains of *B. typhosus* offers an explanation of the equally outstanding potency of this antibody in protection against infection with this type of strain.

The specificity of the reaction between Vi antigen and its corresponding antibody, first established in agglutination and absorption tests, was in the phagocytic experiments again clearly demonstrated by the fact that the Vi antibody was incapable of exercising any sensitizing effect on strains of low virulence, devoid of Vi antigen. On the other hand, the phagocytic tests for the first time showed a summation effect of the separate activities of the Vi and O antibodies. It has been emphasized in a previous paper that in specially designed agglutination tests neither of the two antibodies was found to be capable of enhancing the action of the other. Similarly experiments on passive immunization of mice did not indicate any increase in the protective action of the Vi antibody attributable to the activity of the accompanying O antibodies. Under the more favourable conditions of the phagocytic experiments, however, the summation of the sensitizing effects of each of the two antibodies could be clearly demonstrated (Table II), though the powerful

activity of the Vi antibody tended to overshadow the weak effect of the O antibody.

So far the phagocytic experiments do not indicate any essential difference in the mode of action of Vi and O antibodies. With both types of antibody the presence of complement is required to accomplish efficient sensitization.

The demonstration of high-titre Vi agglutination with the serum of rabbits inoculated with formolized extracts had been welcomed as an advance of considerable practical importance. It seemed that potent Vi antisera for use in the treatment of cases of typhoid fever could be prepared by a simple method of immunization free from the difficulties and risks inherent in the original procedure, according to which horses had to be inoculated with suspensions of virulent bacilli in the living state. This hope had to be abandoned when it was established that Vi antisera produced in response to immunization with formolized extracts were highly deficient in their powers of promoting phagocytosis of virulent strains of *B. typhosus*, and of protecting against attack by strains of this type.

The statement made in a previous paper (Felix, Bhatnagar and Pitt, 1934) that " the presence of formalin in extracts containing the Vi antigen does not destroy its agglutinogenic activity " is correct, but the further statement printed at the end of that paper, viz. : " The use of formolized extracts is suggested for the preparation of relatively potent Vi antiserum," is now shown to have been based upon an erroneous conclusion. It is true that according to the state of knowledge of immunology at that time their conclusion seemed to be a legitimate one. It now appears necessary to voice a warning against the use of formolized Vi antigen in the preparation of a therapeutic antityphoid serum, since Vi antibody of maximum protective potency cannot be produced by this method of immunization.

The extreme susceptibility of the Vi antigen to the action of phenol is a matter of particular importance in the preparation of antityphoid vaccine. The demonstration in this paper of a most harmful effect on the Vi antigen of exposure to the action of phenol even for comparatively very short periods suggests strongly that the immunogenic value of antityphoid vaccines is greatly impaired by this disinfectant. Taken in conjunction with the observation recently published by Stuart and Krikorian (1934), the facts recorded in this communication present a strong case for renewed efforts in the search for some method of preparing typhoid vaccines of maximum immunizing value.

Apart from the immediate bearing these observations have on the special problems of antityphoid inoculation and of serum therapy of typhoid fever, they are obviously of a more general interest. From the theoretical point of view the remarkable fact seems to have been established that antibodies to a single antigen exist, which differ widely in their capacity of functioning in the various immunity reactions in which this particular antigen is involved. Thus the Vi antibody resulting from immunization with formolized Vi antigen is highly deficient in its protective and phagocytosis-promoting functions, whereas its agglutinating activity in no way differs from that of the Vi antibody produced in response to inoculation of the " natural " Vi antigen contained in the living virulent bacilli. In cross-absorption tests with the two varieties of Vi antigen and the corresponding antisera complete mutual absorption is

obtained. From our knowledge of published immunological data the only analogy that suggests itself would appear to be the relationship between haptens and full antigens, though the resemblance between the two phenomena may be apparent rather than real.

Since the alteration of the Vi antigen that is responsible for the formation of an altered Vi antibody is brought about by treatment so trivial as the exposure to 0·2 p.c. formalin, the question arises whether similar effects are not produced in the daily routine of the preparation of vaccines and immune sera. It is very probable that antigenic constituents of the type of the Vi antigen of *B. typhosus* also occur in other bacterial species, and there, too, play some important *rôle* in the complex mechanisms of infection and immunity. Furthermore, it has not yet been investigated adequately whether the phenomenon of "functional deficiency", established in the case of the Vi antibody, has analogies in antibodies to other types of antigen of immunological significance. Renewed research might disclose, at least in some instances, that the widespread use in bacteriological routine of phenol and formalin is not at all conducive to the protective value of vaccines and specific antisera.

SUMMARY.

1. The Vi antibody produced by immunization with suspensions of virulent strains of *B. typhosus* in the living state exerts a powerful phagocytosis-promoting action on strains containing Vi antigen, while it is without any effect on strains devoid of this antigen.

2. The Vi antibody excels the O antibody in the phagocytosis-promoting function in the same way as it does in the protective action against attack by strains of high virulence.

3. There is a summation effect in the sensitizing activities of Vi and O antibodies and their respective modes of action seem to be essentially similar, since both depend on the active participation of complement.

4. The Vi antibody elaborated in response to immunization with formolized Vi antigen is not identical with that resulting from immunization with the "natural" Vi antigen contained in the living virulent bacilli. The phagocytosis-promoting activity and the protective power of the former are much inferior to those of the latter, though there is no difference in the agglutinating properties of the two varieties of antibody.

5. The "functional deficiency" of the altered Vi antibody is discussed, and the apparent analogy to the relationship existing between haptens and antigens is tentatively considered.

6. The extreme susceptibility to phenol of the Vi antigen of *B. typhosus* is demonstrated.

7. The necessity of using the "natural" Vi antigen from live bacilli for the preparation of therapeutic antityphoid serum of high potency is emphasized.

8. The possible application of these facts to other bacterial species is suggested.

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ABBREVIATIONS USED IN REFERENCES.

The abbreviations given in the second edition of the 'World List of Scientific Periodicals' are now used in place of those given in the first edition. The chief difference is that adjectives are no longer given initial capitals in English.

ERRATA.

(Vol. XVI, pp. 204 and 214.)

The legend for Diagram 2 should read "Lower curve: Filtrate prepared with toxigenic subculture of strain No. 3203. Upper curve: Filtrate prepared with non-toxigenic subculture of strain No. 3203."

The legend for Diagram 9 should read "Lower curve: Extract from untreated filtrate. Upper curve: Extract from charcoal-treated filtrate."